#P3-194: ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH.

INCSTAR®
Science, Technology and Research

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IMU-3282

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Introduction

Intact PTH secretion is equisitely controlled by serum ionized calcium in a classic negative feedback loop. Sensitive calcium receptors on parathyroid chief cells detect very slight changes in the concentration of Ca^{2+} and secrete intact PTH in response to low concentrations. Total serum calcium exists in three phases in dynamic equilibrium: protein bound (mainly albumin, Ig), complexed (phosphate, bicarbonate, citrate), and ionized calcium.

The clinical utility of a PTH measurement, in combination with a serum calcium determination, is in diagnosis of primary hyperparathyroidism, management of uremic or seconday hyperparathyroidism, and diagnosis of hypoparathyroidism.

Previously characterized serum PTH heterogeneity includes the intact PTH (1-84) active peptide hormone, and inactive C-terminal fragments with N-termini between PTH (34-43). Thus, strategies for immunoassay evaluation of intact PTH concentrations in serum involved a solid phase antibody present in excess to overcome interfering C-terminal fragments and thus detecting intact PTH only using a N-terminally directed reporter antibody.

N-Terminal truncations yielding PTH species larger than PTH 34-84 have been isolated from human parathyroid cell monolayer.C-terminal fragments have been detected in extracts of parathyroid gland, and parathyroid effluent samples collected from hypercalcemic patients with hyperparathyroidism. Based on immunoassay value differences observed by our group and PTH immunoreactivity by Brossard et.al. (JCEM 81(11): 3923-3929) there appears to be differences in immunoassay values caused by fragment recognition in some commercially available intact PTH assays.

Evidence for a circulating inhibitor to PTH has arisen from studies of patients with pseudohyperparathyroidism. Despite elevated levels of immunoreactive PTH, bioactive PTH was normal when tested in bioassay systems. Plasma from these patients has been shown to diminish the biological activity of exogenous PTH in these *in vitro* bioassays.

To determine what PTH molecular forms circulate, and elucidate possible biological activity, a detection system was developed to elucidate the concentrations of intact PTH and the novel large molecular weight fragments of PTH, without interference or recognition of the hepatically generated C-terminal fragments. Data generated was evaluated for diagnostic potential with regard to primary hyperparathyroidism, and correlation to serum calcium in primary and uremic hyperparathyroidism.

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Abstract

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

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PTH Values inUremic Hyperparathyroidism

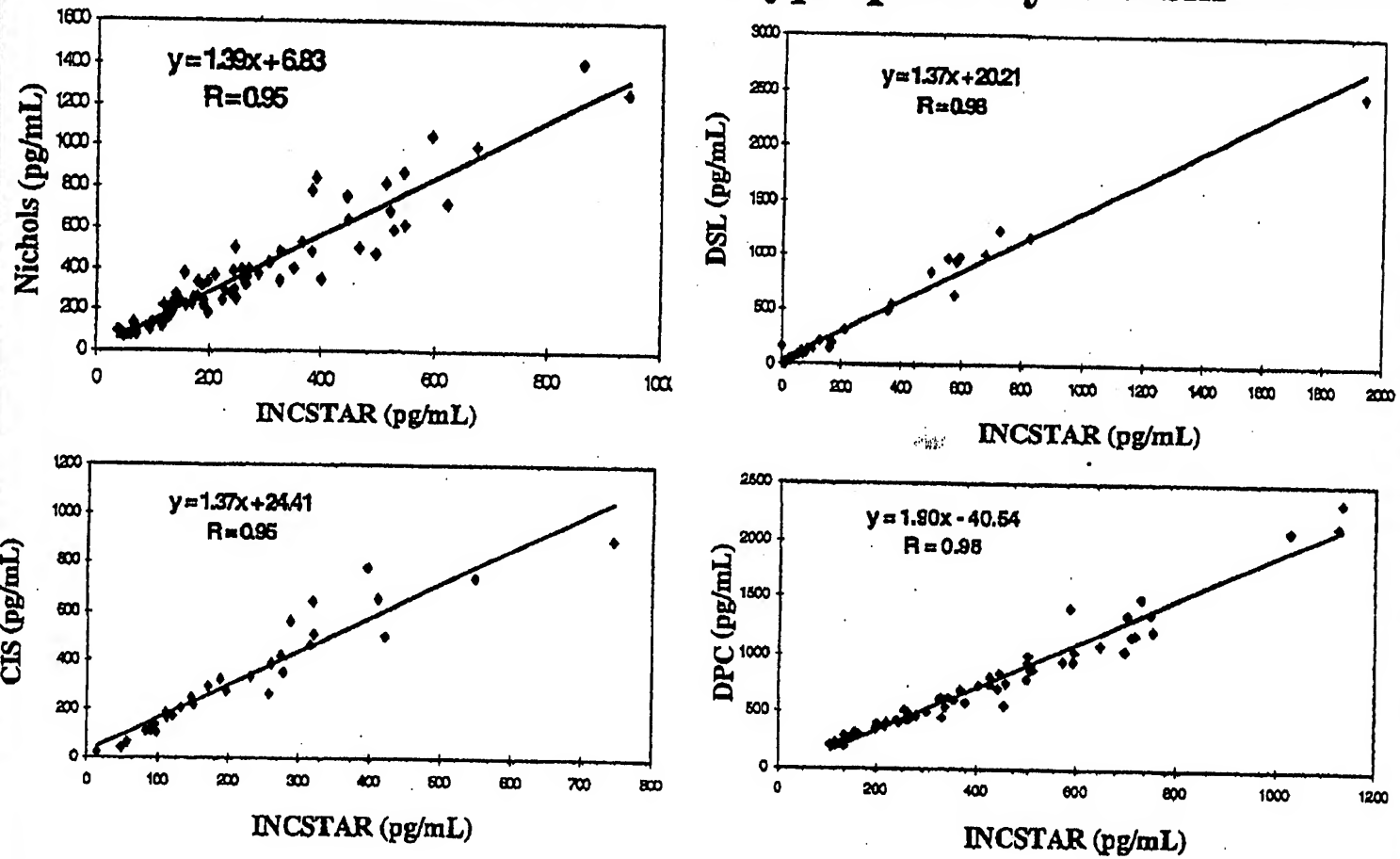

Figure 1. Correlations of several commercially available intact PTH assays verses the INCSTAR N-tact PTH SP intact PTH assay. All of the commercial assays evaluated indicate much greater cross-reactivity to the novel PTH fragments.

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INCSTAR PTH Values vs. Nichols PTH Values

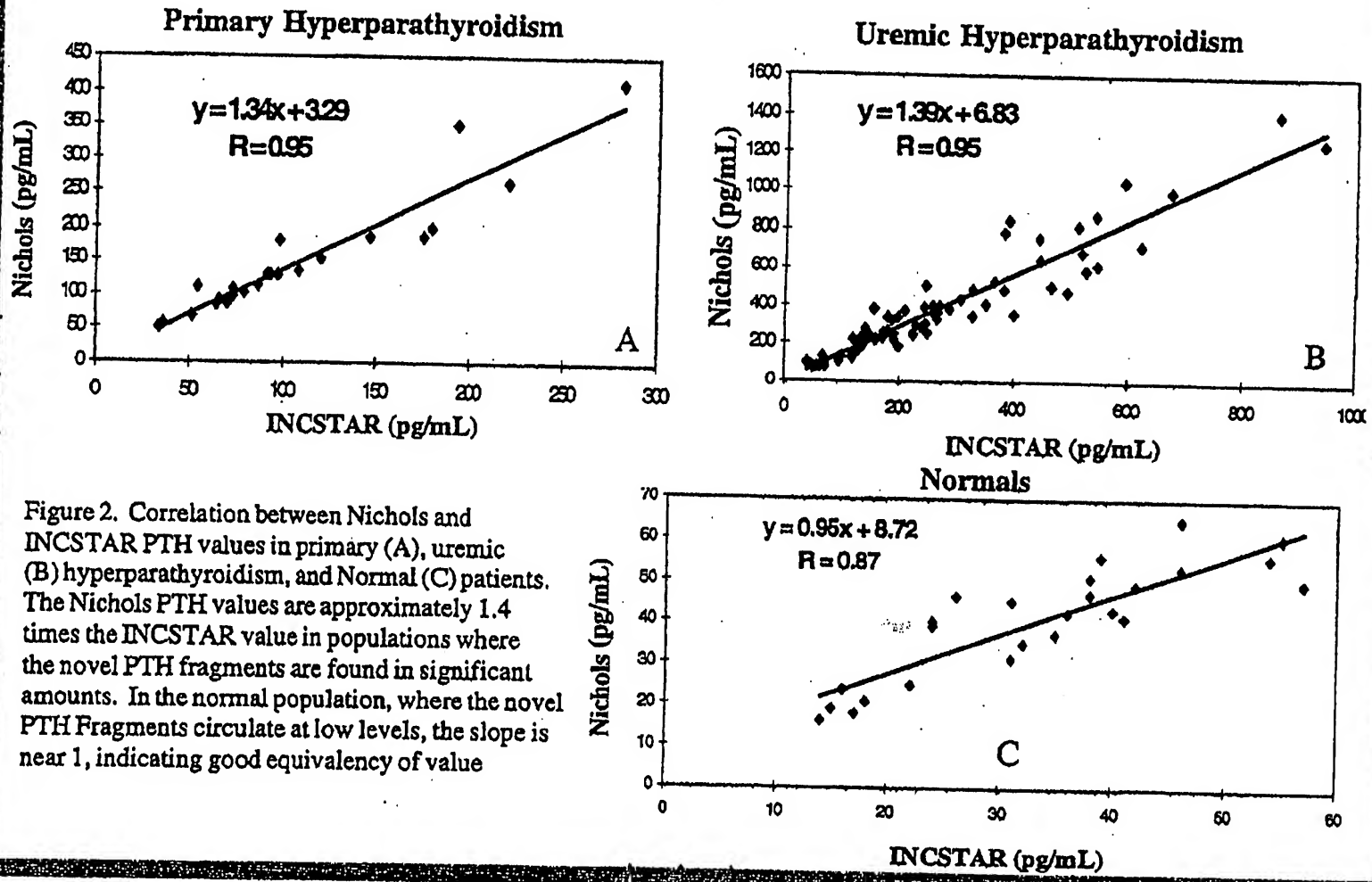
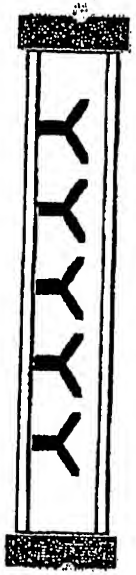


Figure 2. Correlation between Nichols and INCSTAR PTH values in primary (A), uremic (B) hyperparathyroidism, and Normal (C) patients. The Nichols PTH values are approximately 1.4 times the INCSTAR value in populations where the novel PTH fragments are found in significant amounts. In the normal population, where the novel PTH Fragments circulate at low levels, the slope is near 1, indicating good equivalency of value

Method of Isolation of PTH Molecular Forms



Delipidated EDTA plasma is loaded on the column. PTH Molecular forms containing all or parts of the (39-84) region are captured. The specifically bound protein is eluted with 0.2 M glycine pH 2.5

Anti-PTH(39-84) Immunoextraction Column

The protein eluted from the immunoextraction step is loaded onto a C_{18} reverse-phase HPLC column. The column resolves homologous proteins by size. 2-60% - 0.1% TFA/Acetonitrile : 0.1% TFA/dH20 over 58 minutes 1%/minute.

C_{18} Reverse Phase HPLC Column

HPLC Fractions were tested for PTH immunoreactivity

Figure 3. Method of isolation of PTH molecular forms.

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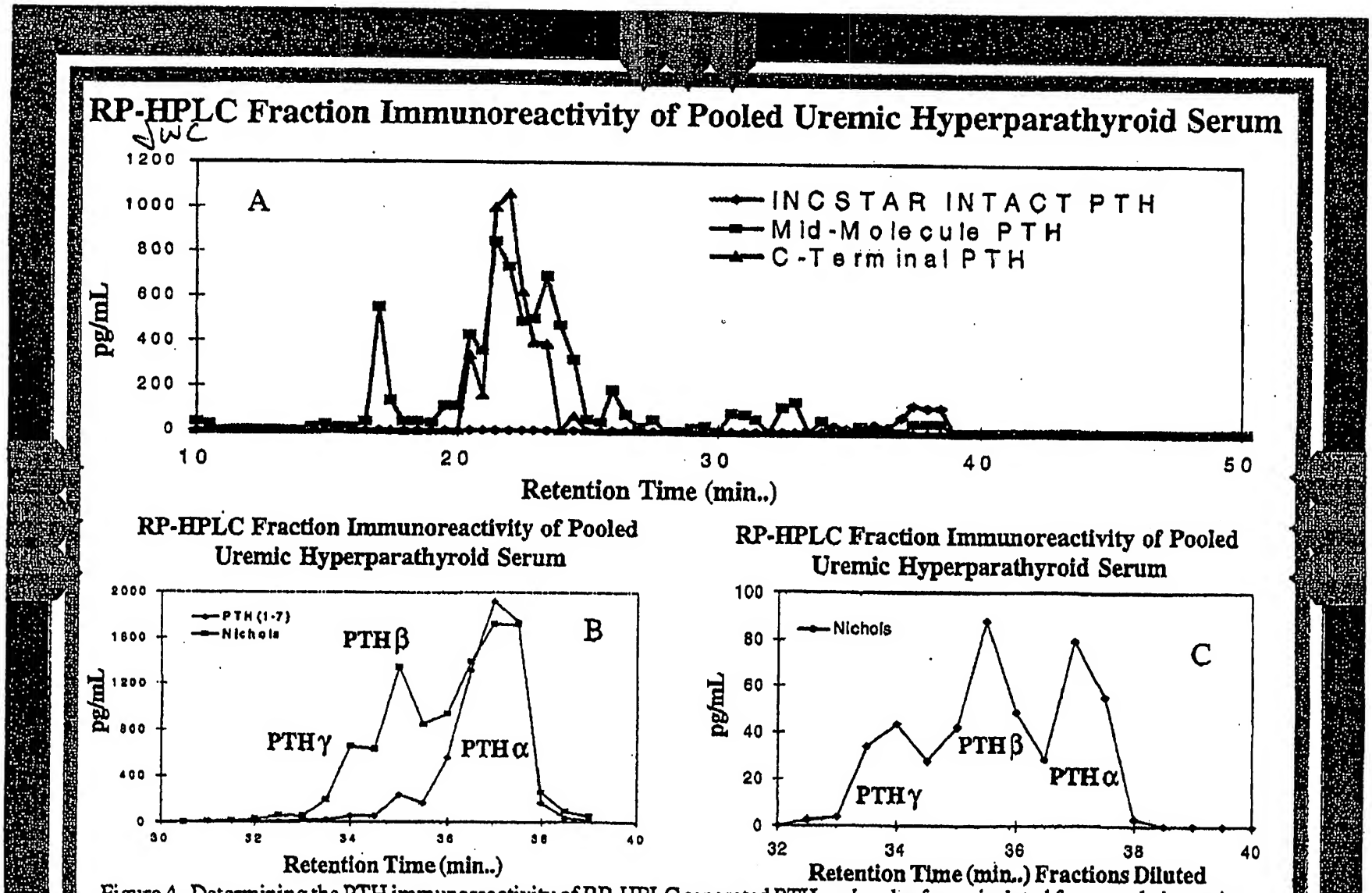

Figure 4. Determining the PTH immunoreactivity of RP-HPLC separated PTH molecular forms isolated from pooled uremic hyperparathyroid serum. The PTH (1-7) directed tracer antibody (B) clearly shows that the fragments are N-terminally truncated. Both B and C show two novel PTH molecular forms (PTH β,γ) in addition to intact PTH (PTH α).

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PTH Molecular Forms

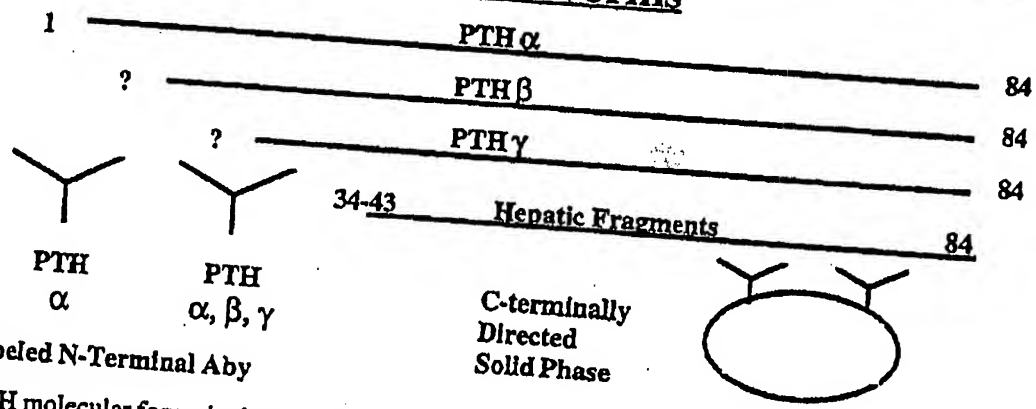

Figure 5. Defining PTH molecular forms isolated by RP-HPLC and the detection system used to estimate their concentration. These fragments have N-termini that extend beyond amino acid 34, and do not include hepatically generated fragments.

PTH Ratio

$$\frac{[\text{PTH } \alpha, \beta, \gamma] - [\text{PTH } \alpha]}{[\text{PTH } \alpha]} = \frac{\text{PTH } \beta,\gamma}{\text{PTH } \alpha}$$

This calculation yields a ratio of the two novel C-terminal Fragments / Intact PTH

The hepatically generated C-terminal fragments are NOT evaluated in this system

Figure 6. Defining the PTH Ratio to be the concentrations of the two novel PTH fragments to the concentration of intact PTH

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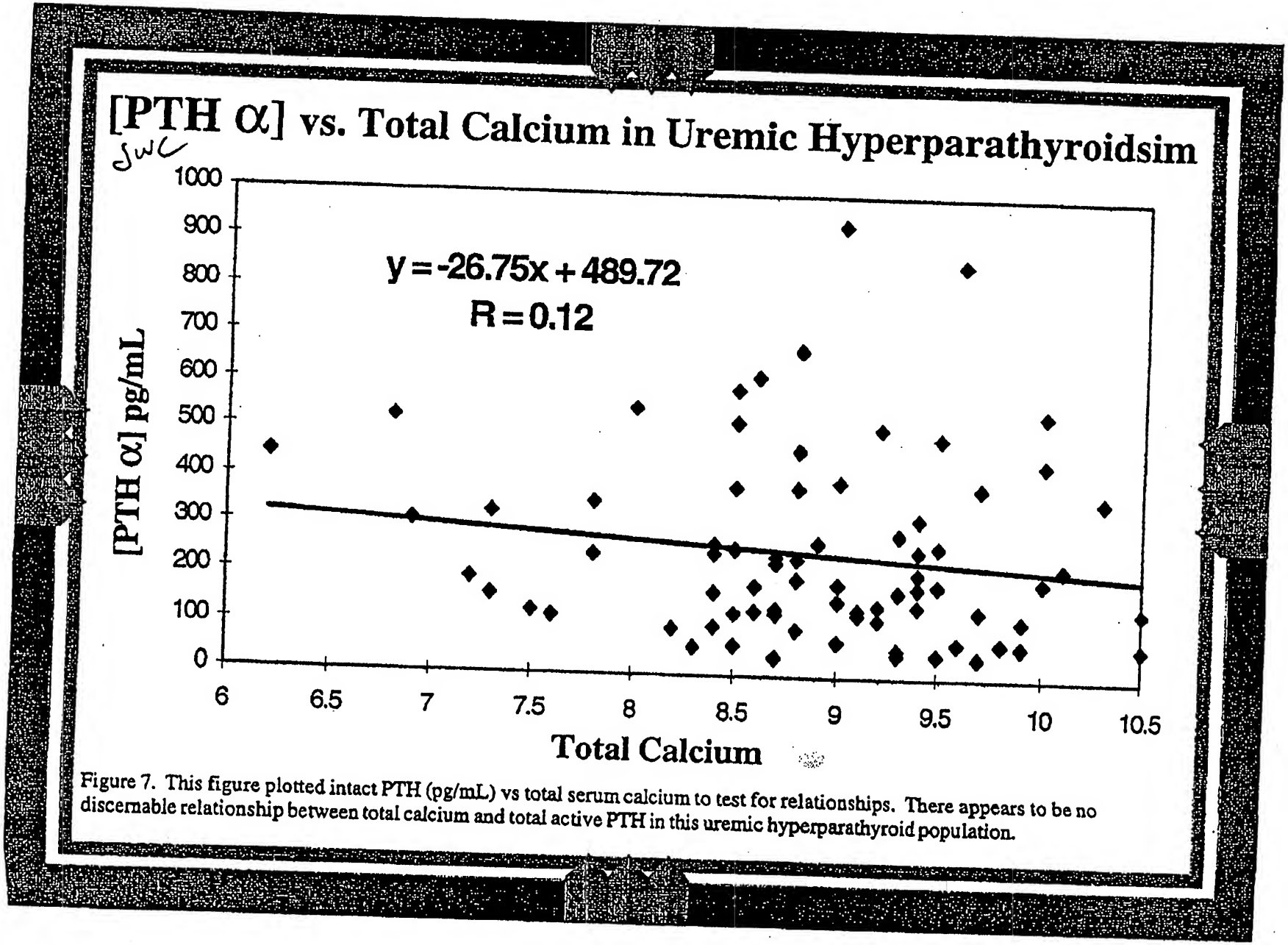

Figure 7. This figure plotted intact PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernable relationship between total calcium and total active PTH in this uremic hyperparathyroid population.

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PTH[α , β, γ] vs. Total Calcium in Uremic Hyperparathyroidism

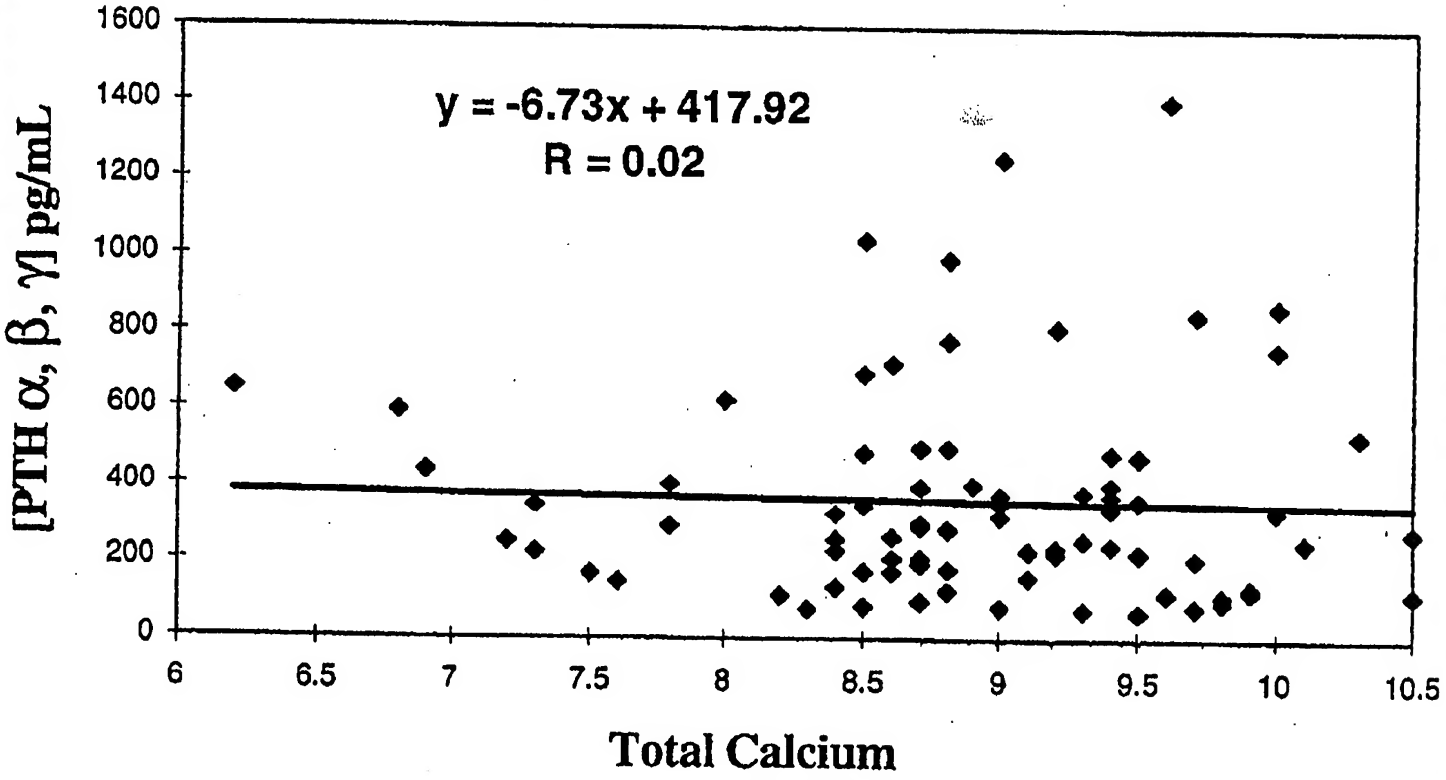

Figure 8. This figure plotted Total active PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernablerelationship between total calcium and total active PTH in this uremic hyperparathyroid population.

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PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Uremic Hyperparathyroidism

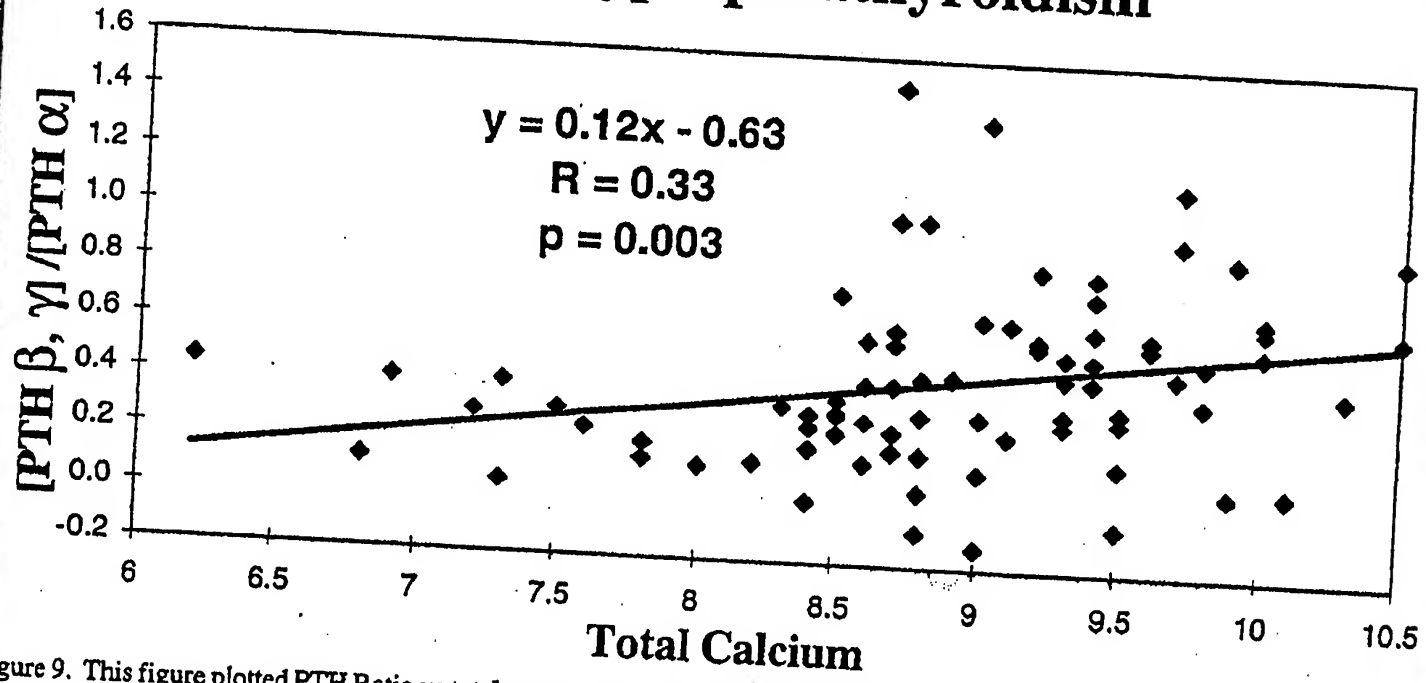

Figure 9. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, the corresponding serum calcium approaches normalization in uremic hyperparathyroidism even though most of these samples have elevated intact PTH levels. This trend is opposite to that in primary hyperparathyroidism. Endogenous and exogenous factors, such as calcitriol therapy, complicate calcium homeostasis in uremic hyperparathyroidism, and thus cause and effect conclusions cannot be made in this population without more information. However, this is consistent with an inhibitory effect of these fragments to PTH in these patients.

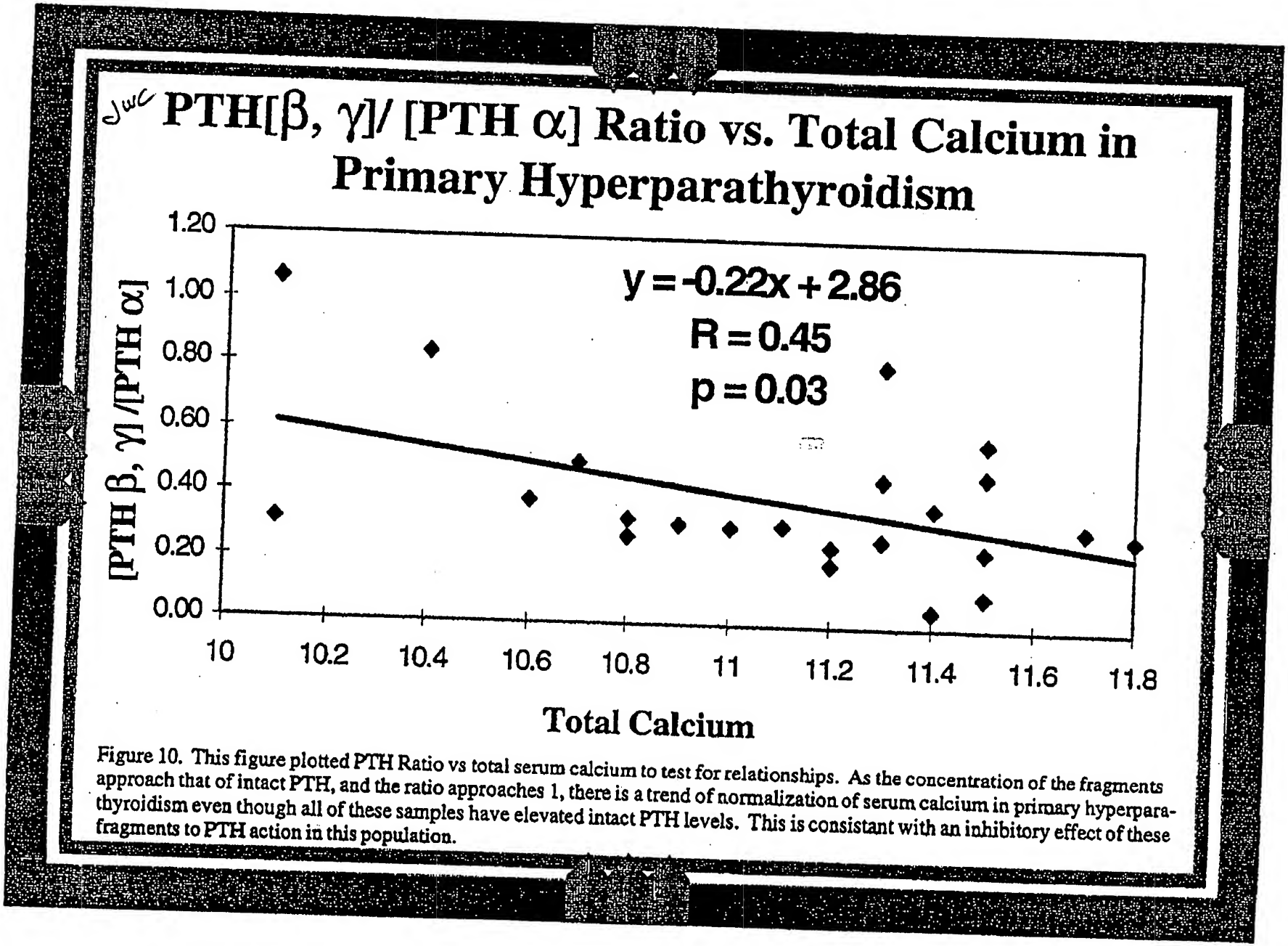

Figure 10. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, there is a trend of normalization of serum calcium in primary hyperparathyroidism even though all of these samples have elevated intact PTH levels. This is consistant with an inhibitory effect of these fragments to PTH action in this population.

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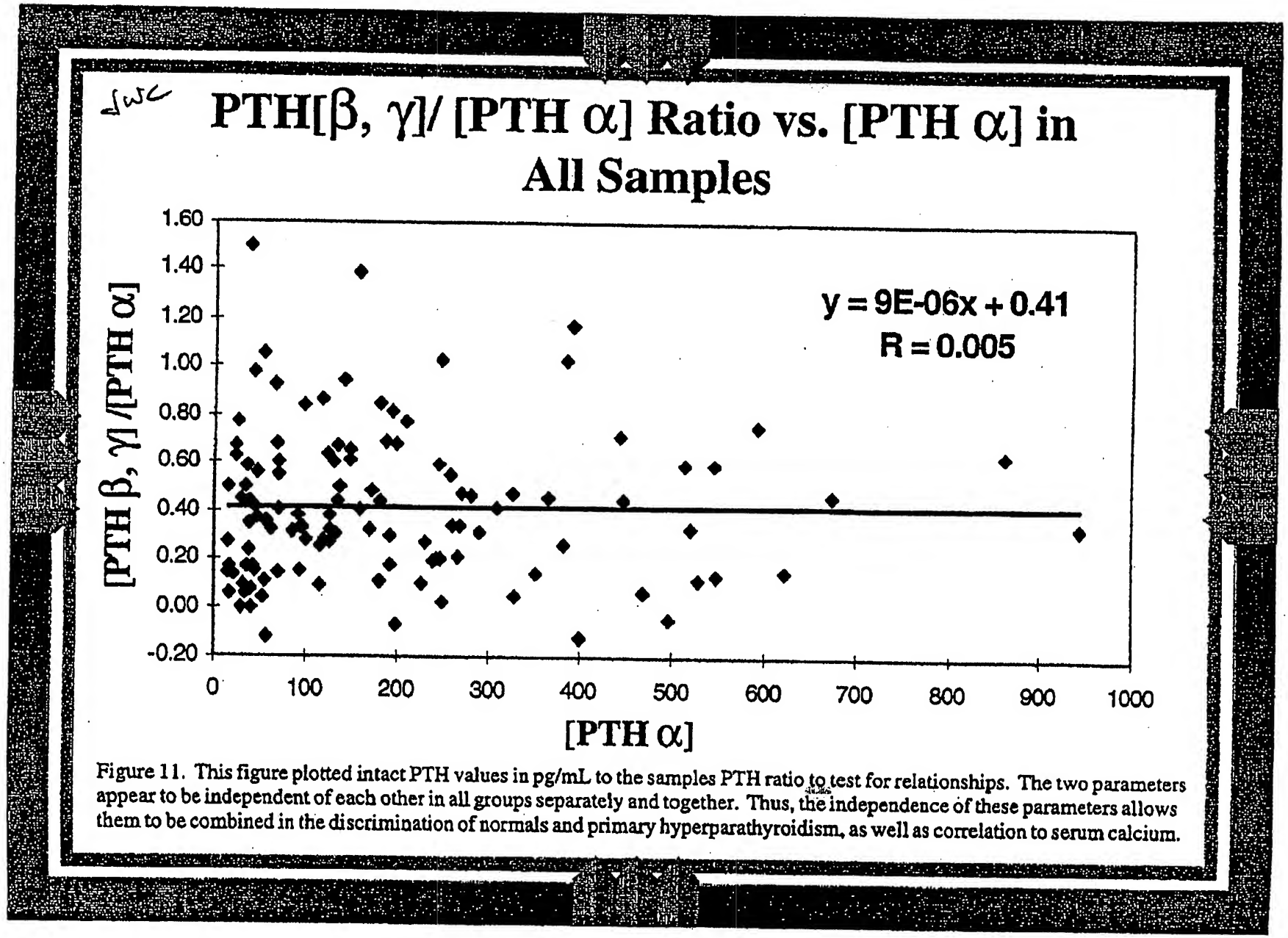

Figure 11. This figure plotted intact PTH values in pg/mL to the samples PTH ratio to test for relationships. The two parameters appear to be independent of each other in all groups separately and together. Thus, the independence of these parameters allows them to be combined in the discrimination of normals and primary hyperparathyroidism, as well as correlation to serum calcium.

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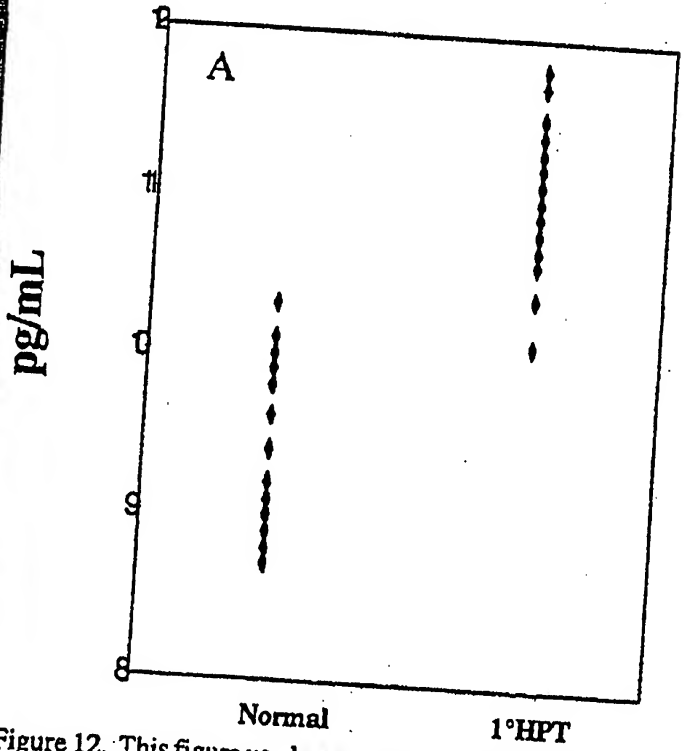
Discrimination Power of Total Calcium



Discrimination Power of PTH

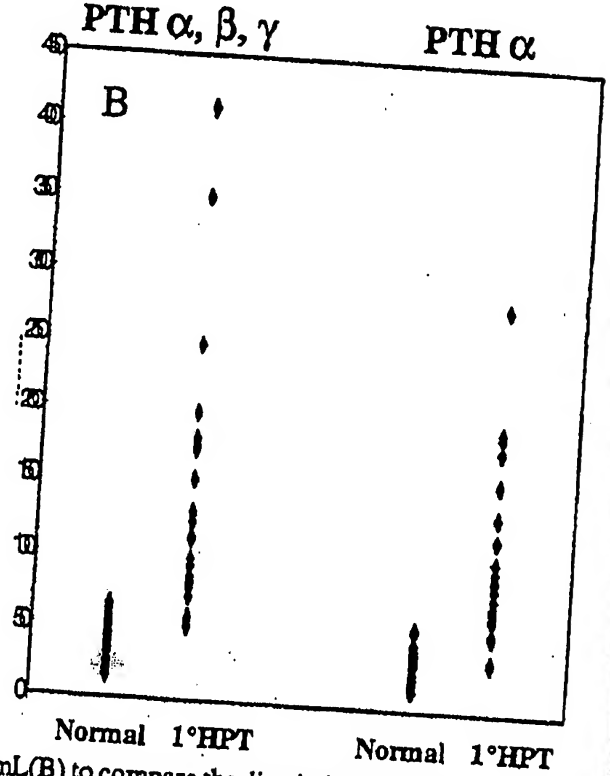


Figure 12. This figure used serum total calcium (A) and PTH values in pg/mL(B) to compare the discrimination power of calcium and PTH measurement in the separation of a normal and primary hyperparathyroid population. There is clearly significan overlap of the populations using both calcium and PTH determinations separately. These parameters are typically combined in a clinical situation to provide optimal discrimination, but which PTH parameter is the best is evaluated in Figure 13.

Discrimination Power of PTH measurements + Total Calcium

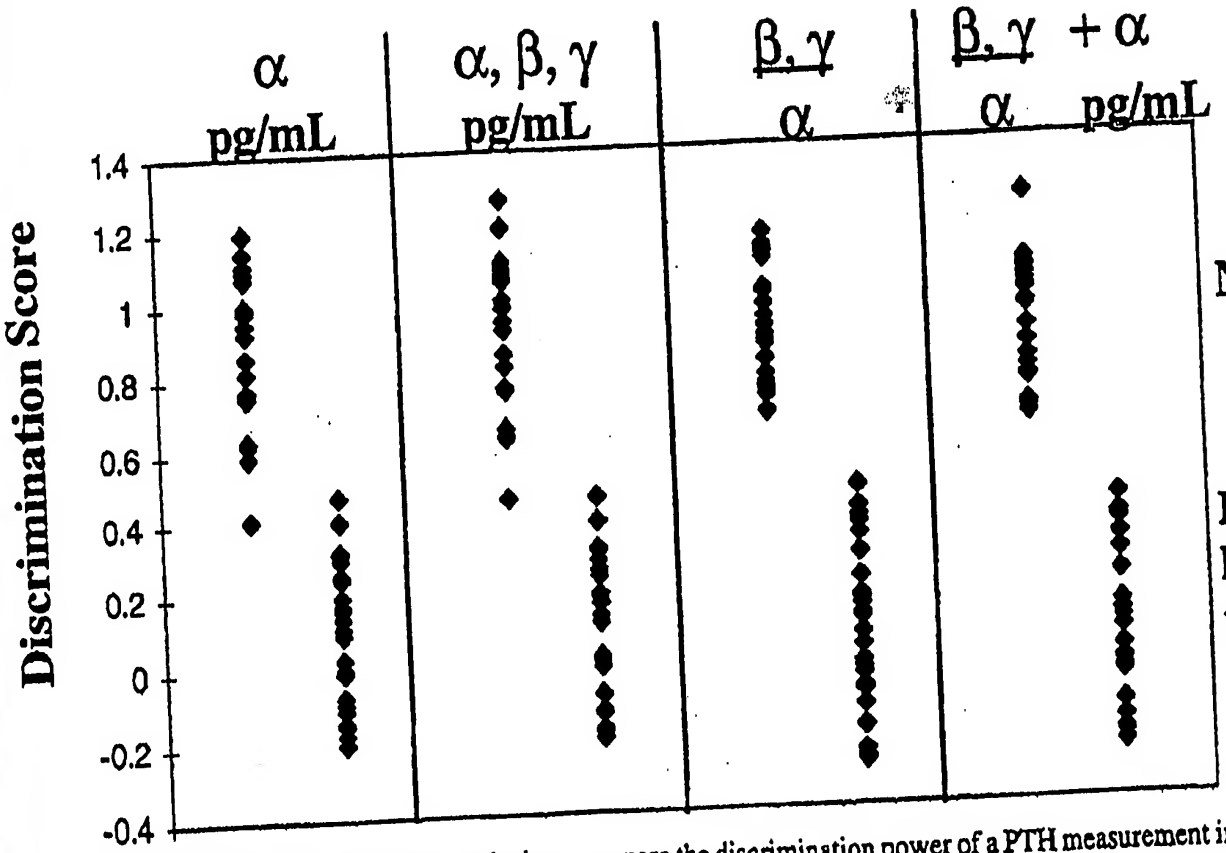
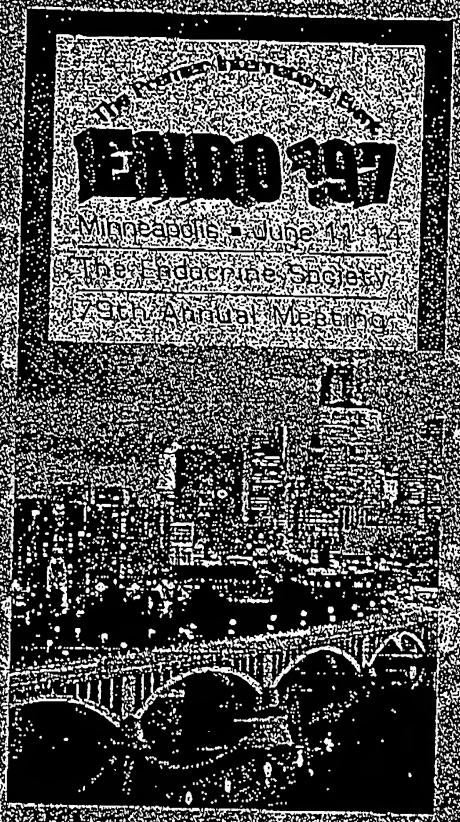


Figure 13. This figure used regression analysis to compare the discrimination power of a PTH measurement in addition to a total calcium determination in the separation of a normal and primary hyperparathyroid population. The combination of a PTH measurement with a serum calcium determination is the common clinical situation in diagnosis of primary hyperparathyroidism. The PTH ratio allows clear distinction of borderline cases into the respective populations.

CONCLUSIONS

- There appears to be PTH heterogeneity found in circulation in patients with primary and uremic hyperparathyroidism beyond what has been characterized thus far.
- These novel PTH fragments appear to be N-terminally truncated and are reported in some commercially available intact PTH assays.
- As levels of the fragments approach those of Intact PTH, serum calcium trends toward normalization in patients with primary and uremic hyperparathyroidism
- Based on the data and references, we speculate that these fragments are a strong candidate for being a competitive inhibitor to intact parathyroid hormone action at the PTH/PTHrP receptor in patients with primary hyperparathyroidismand mediate its biological activity.
- Complete characterization of PTH molecular forms provides a better correlation to serum calcium, and provides greater clinical discrimination between primary hyperparathyroid and normals.

PROGRAM & ABSTRACTS



79th ANNUAL MEETING

JUNE 11-14, 1997

MINNEAPOLIS, MINNESOTA

THE ENDOCRINE SOCIETY

P03-194
Friday June 13, 1997
PTH and Calcium [BASIC - Poster Session] (Exhibit Hall 1&2)

Isolation and characterization of large molecular weight fragments of PTH

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Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5 M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

Biochemistry: other
Parathyroid hormone

COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTHSP AND NICHOLS INTACT PTH ASSAYS.

Todd Jensen, Jon Spring, and John Colford (INCSTAR Corporation, Stillwater, MN 55082)

ABSTRACT

The N-terminus of the PTH molecule is required for PTH receptor interaction. It has been shown that cleavage of three amino acids from the N-terminus will inactivate PTH activity *in vivo*. Specificity is an advantage of a two antibody assay format of an IRMA. An N-terminal truncated circulating inhibitor to PTH has been hypothesized, but not found. Both the INCSTAR Intact PTHSP™ assay and the Nichols Allegro™ Intact PTH claim to be specific for the intact molecule. In a comparison between the two kits, patients with renal failure were assayed. The results showed that the Nichols assay results to be an average of 1.52 times the INCSTAR assay value (n = 14). Intact 1-84 PTH was spiked into clarified human serum and assayed in both kits. The results showed that the Nichols assay results to be an average of 1.07 times the INCSTAR assay value (n = 12).

Both assay tracers are directed to the N-terminal region of the intact PTH molecule. To compare specificity of the two assay tracers, we used a (7-84) C-terminal fragment. While not a defined byproduct of PTH metabolism *in vivo*, it could provide insight to the degree the population of antibodies in the tracer are directed to the N-terminus. Based on weight, target levels of 1500, 1000, and 500 pg/ml (7-84) PTH C-terminal fragment were spiked into clarified human serum. the Nichols assay had values of 520, 374, and 245 pg/ml, respectively. The INCSTAR assay had values of 220, 167, and 114 pg/ml, respectively. This showed that the Nichols results to be an average of 2.25 times the INCSTAR assay value (n = 14).

The INCSTAR tracer was substituted into the Nichols assay and the (7-84) PTH spiked serum samples were assayed, with the hybrid assay reporting 258, 191, and 121 pg/ml, respectively. This suggests that the INCSTAR tracer gives the INCSTAR assay greater specificity for the intact PTH molecule than the Nichols assay. These results also suggest the presence of a C-terminal fragment of PTH in renal samples that has not been currently identified.

INTRODUCTION

Recently, it has been reported that there is a non-(1-84) molecular form of PTH found in normals and renal samples that is immunoreactive in the Nichols Allegro™ Intact PTH assay (Brossard et al, Proc. Int. Cong. Endo, Vol2, OR60-5). The PTH molecule is degraded in the parathyroid gland itself and also in the liver. The breakdown products don't have most of the N-terminal 27 amino acids and lack hypercalcemic, hypocalciuric, and phosphaturic activity. These fragments have a considerable half-life and accumulate in circulation up to 20 times the concentration of the intact hormone. Their presence makes the immunologic assessment of the biologically active hormone difficult.

The hepatic fragments are generated when enzymes on the surface of Kupffer cells cleave the hormone in its 34-43 region. These fragments are formed at a rate dependent largely on the concentration of intact PTH. The N-terminal fragment is at very low concentrations in serum, suggesting a quick and thorough removal from circulation. Fragments generated in the parathyroid gland are truncated at both ends of the molecule. These fragments are not currently defined. In hypercalcemic patients of a non-parathyroid origin, the negative feedback mechanism will suppress PTH release to almost nothing, but the parathyroid gland will continue to release truncated fragments. In patients that have nonparathyroid forms of hypercalcemia, these fragments have been detected by a carboxy- terminal RIA but the N-terminus was not defined. All PTH fragments are removed from circulation by the kidney. In cases of renal insufficiency, fragments accumulate.

There appear to be differences in the values for renal samples among the Nichols Allegro™, DSL Active™, and INCSTAR N-tact® PTHSP assays. To explore these differences, PTH (1-84) and (7-84) immunoreactivity was validated between the assays. If PTH (1-84) equivalence is shown, renal sample differences will be significant. The results will be compared to reported non-(1-84) PTH immunoreactivity.

Dilution Linearity Validation

Pat. ID#	46	46 1:2	46 1:4
Nichols	1142	573	277
DSL	1233	707	378
INCSTAR	725	354	159
EXP(1-7)	860	383	158
EXP(7-84)	680	343	158

% Recovery	
46 1:2	46 1:4
100	97
115	123
98	88
89	74
101	93

Pat. ID#	48	48 1:2
Nichols	877	485
DSL	983	546
INCSTAR	593	278
EXP(1-7)	613	300
EXP(7-84)	533	253

% Recovery
48 1:2
111
111
94
98
95

Pat. ID#	49	49 1:2
Nichols	143	67
DSL	143	69
INCSTAR	107	51
EXP(1-7)	102	44
EXP(7-84)	104	59

% Recovery
49 1:2
94
96
95
87
112

PTH(7-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(7-84)
1	272	217	193	238
2	515	395	296	393
3	1262	1168	816	1094
4	1720	1623	1059	1693

$p<$ 0.0004

INCSTAR Tracer is unique
Nichols, DSL, and EXP (7-84)
are not shown different

PTH(1-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)
1	175	168	175	201	182
2	188	184	222	263	218
3	696	772	620	648	615
4	1058	1152	1045	1074	988

$p=$ 0.45

No Observed Difference

PTH Tracer Specificity.

	NSB	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	20	5419	40	1452	3643	3294	8	0	12	14	19
DSL	559	11627	415	5585	8922	6916	556	364	424	319	278
Nichols	195	2982	139	968	2606	2136	153	138	139	220	142

Yes is a signal 2 SD above Background

	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No
DSL	Yes	No	Yes	Yes	Yes	No	No	No	No	No
Nichols	Yes	No	Yes	Yes	Yes	No	No	No	No	No

Values from two separate assays confirming differences in (7-84) Spiked serum values between Nichols and INCSTAR

FRAGMENT(7-84) SPIKES (pg/mL)

	Target Value*	INCSTAR Assay	Nichols Assay	DIFFERENCE
Assay 1	1500	511	774	-262
	1000	366	577	-211
	500	220	346	-125
Assay 2	1000	220	520	-300
	400	167	374	-207
	250	114	245	-131

* - Value based on assumption of 100% purity and quantitative transfer in dilution

MEAN=	-206.0	
Variance=	4835.4	
Standard Error=	28.39	
***t*=**	7.26	
***p*=**	0.00078	Significant difference

Comparison of (1-84) Spiked serum values and controls between Nichols and INCSTAR

		INTACT (1-84) (pg/mL)		
		INCSTAR	Nichols	DIFFERENCE
Serum Spikes	**spike 1**	2805	2883	-77
	spike 2	1617	1712	-95
	spike 1 Diluted 1:10	273	236	37
	spike 2 Diluted 1:10	157	152	5
	spike 3	1656	1589	67
	spike 4	1740	1724	16
	spike 3 Diluted 1:10	183	212	-29
	spike 4 Diluted 1:10	164	190	-26
Kit Controls	**NJ**	36	31	6
	NK	265	214	51
	(INC)L1	33	34	-1
	(INC)L2	415	330	85
	(INC)L1	49	41	8
	(INC)L2	447	426	21

MEAN of Ln*=	0.0456
Standard Error of Ln*=	0.0330
t=*	1.38
p=*	0.1896
NOT a significant difference	

* - Data generated from the Natural Log transformed data. This transformation is necessary due to the large range the data. It reduces the chances for error.

Patient Sample Comparison

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)	
1	196	197	170	195	163	renal failure
2	17	8	15	20	14	kidney stones
3	920	943	580	568	478	renal failure/hyperPTH
4	176	144	161	220	148	n/a
5	17	11	12	15	15	renal failure
6	375	540	366	542	341	renal failure
7	66	75	51	60	60	renal failure
8	28	25	26	26		renal failure
9	50	59	35	34	38	renal failure
10	89	104	80	79	75	renal failure
11	1043	1160	825	988	831	renal failure
12		0	6			renal failure
13	166	209	126	126	117	bone disease
14	78	99	69	115	68	renal failure
15	31	35	28	32	31	renal failure
16	17	11	17			kidney stones
17	930	959	552	588	473	renal failure/hyperPTH
18	202	192	166	174	172	renal failure
20	143	159	0	141	92	renal failure
21	121	120	69	63	72	renal failure
22	773	850	498	604	523	renal failure/hyperPTH
23	34	27	24	28	23	renal failure
24	104	105	84	85	79	renal failure
25	11	7	12	12	11	renal failure
26	293	316	211	194	186	renal failure
27	105	135	87	81	86	renal failure
28	473	495	353	380	370	renal failure
29	28	22		27	24	n/a
30	74	94	71	70	62	renal failure
31	69	88	59	48	55	renal failure
32	892	1000	677	787	651	renal failure/hyperPTH
33	14	6	13		18	renal failure
34	648	629	575	526	544	renal failure
35	2043	2476	1942	2230	1904	renal failure/hyperPTH
46	1142	1233	725	860	680	renal failure/hyperPTH
47	31	29	31		31	kidney stones
48	877	983	593	613	533	renal failure/hyperPTH
49	143	143	107	102	104	renal failure
50	49	42	33	31	35	renal failure

Significantly Different Groups $p<$ 0.0001

There are two sharp, well separated groups, by Tukey's test

Group 1
Nichols
DSL

Group 2
INCSTAR
EXP(1-7)
EXP(7-84)

CONCLUSIONS

In the Nichols Allegro™ Intact PTH, DSL Active™ PTH and INCSTAR N-tact® PTHSP equivalence of values for samples with clearly defined 1-84 was validated using spikes throughout the standard range, and dilutions to verify values. There is no significant difference for PTH(1-84) values between the three kits ($p = 0.45$). Values for renal samples show a significant difference between the INCSTAR Assay and the other two kits (Nichols and DSL) which report up to twice the INCSTAR value ($p < 0.0001$). PTH (7-84) immunoreactivity was the same for the Nichols, DSL, and one experimental INCSTAR tracer. Two differently purified tracers from INCSTAR with significantly different PTH(7-84) immunoreactivity did not report different values for renal samples, so PTH(7-84) immunoreactivity itself does not elucidate non-(1-84) PTH immunoreactivity. These results suggest that the differences between INCSTAR assay values and the other two kits (Nichols and DSL) is the differing levels of cross-reactivity to fragments present in renal samples, likely of parathyroid gland origin. Based on specificities of each tracer, the N-termini of the fragment(s) reside inside the PTH (7-28) region, and likely end in the PTH (13-28) region. If these data are confirmed in sequencing the fragment, it would not have PTH-receptor mediated biological activity.

Materials and Methods

Assaying of Spiked samples and patient samples:

Respective assay protocols were followed for the collection of data. The PTH(1-84) and PTH(7-84) were purchased from BACHEM. The serum matrix used to spike into was low pH stripped, charcoal stripped, defibrinated, delipidated normal serum. The peptides were dissolved in 5% acetic acid, and serially diluted in the serum matrix to target values within each assays standard range. The spikes were targeted to values based on weight on the vial label, and the entire contents were dissolved with no quantitative assessment of a concentration. All samples were assayed side by side in each kit. The neat value for the serum matrix without spiking was 0.0 pg/mL in both assays. Both Nichols and INCSTAR kit controls were assayed in each others kits and reported as (1-84) spikes.

Peptide Coated Wells for the Specificity Screen:

(modified from Ball et. al., J. Imm. Meth. 171(1994) 37-44)
Briefly: Poly Lys:Tyr (1:1) is coated in PBS to microtiter plates, washed, then the Lys amines are activated with glutaraldehyde. The plates are washed again, and peptides are added to specific wells and left to conjugate over night. The Shiff bases are reduced to primary amines with sodium cyanoborohydride, then the wells are blocked to prevent NSB.

PATENT
Docket No. 532212801100

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Marian Christopher

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Reexamination of:

Cantor, et al.

Patent No.: 6,689,566 B1

Issue Date: February 10, 2004

Assignee: Scantibodies Laboratory, Inc.

Examiner: To be assigned

DECLARATION OF JOHN COLFORD

MS Ex Parte Reexam
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, John Colford, declare as follows:

1. I am one of the listed co-authors of following documents:

   - Colford J, Salvati M, MacFarlane G, Sokoll L, and Levine M. (1997), entitled "Isolation and Characterization of Large Molecular Weight Fragments of PTH," #P3-194 79th Annual Meeting of the Endocrine Society Program and Abstracts, Minneapolis, MN, U.S.A. (Colford 1997 Abstract) (Ex. 1);
   - Presentation material of Colford 1997 Abstract (Colford 1997 Presentation) (Ex. 2); and

The poster from the 1996 Annual Meeting of the Endocrine Society, San Francisco, CA, U.S.A., Todd Jensen, Jon Spring, and John Colford, entitled "COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTH SP AND NICHOLS INTACT PTH ASSAYS" (Jensen 1996 Poster) (Ex. 3).

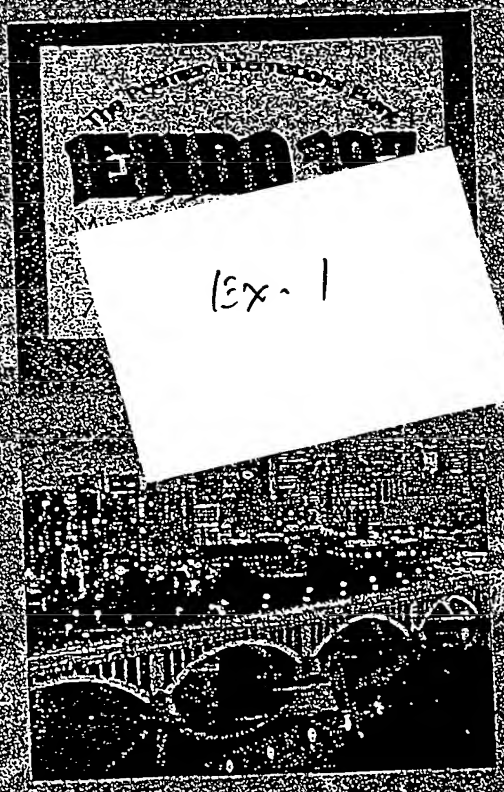
2. I have personal knowledge of the subject matters described in the above documents.

3. Both the Colford 1997 Abstract and the Colford 1997 Presentation refer to a "PTH (1-7) antibody." (Ex. 1 at page IMU-3281 and Ex. 2 at pages IMU-3283, IMU-3284 and IMU-3288.) Jensen 1996 Poster refers to "EXP (1-7)." (Ex. 3 at pages 4, 5 and 9.) The "EXP (1-7)" in the Jensen 1996 Poster refers to an immunoassay for PTH in which the "PTH (1-7) antibody" referred to in the Colford 1997 Abstract and the Colford 1997 Presentation was used.

I declare under penalty of perjury of the laws of the United States that the foregoing is true and correct and that this declaration was executed at HAMPTON, Minnesota on August 19, 2005.

John Colford

PROGRAM & ABSTRACTS



Ex. 1

79th ANNUAL MEETING

JUNE 11-14 1997

MINNEAPOLIS, MINNESOTA

THE INTERURBAN SOCIETY

EXHIBIT 1

P03-194
Friday June 13 , 1997
PTH and Calcium [BASIC - Poster Session] (Exhibit Hall 1&2)

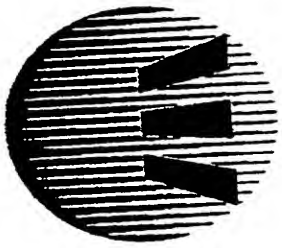
Isolation and characterization of large molecular weight fragments of PTH

J.W. Colford[1], M. Salvati[1], G. MacFarlane[1], L.J. Sokoll[2], M.A. Levine[2]

[1] *INCSTAR Corp., Stillwater, MN, USA*
[2] *The Johns Hopkins School of Medicine, Baltimore, MD. USA*

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5 M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding. while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

Biochemistry: other
Parathyroid hormone



THE ENDOCRINE SOCIETY

Program & Abstracts

Ex. 2

79th Annual Meeting

June 11-14, 1997 Minneapolis, Minnesota

EXHIBIT 2

IMU-3280

P3-194

ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH. JW Colford-[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins School of Medicine, Baltimore, MD 21205.

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

JWC #P3-194: ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH.

JW Colford*[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins Medical Institutions, Baltimore, MD 21205.

IMU-3282

JWL

Introduction

Intact PTH secretion is equisitely controlled by serum ionized calcium in a classic negative feedback loop. Sensitive calcium receptors on parathyroid chief cells detect very slight changes in the concentration of Ca^{2+} and secrete intact PTH in response to low concentrations. Total serum calcium exists in three phases in dynamic equilibrium: protein bound (mainly albumin, Ig), complexed (phosphate, bicarbonate, citrate), and ionized calcium.

The clinical utility of a PTH measurement, in combination with a serum calcium determination, is in diagnosis of primary hyperparathyroidism, management of uremic or seconday hyperparathyroidism, and diagnosis of hypoparathyroidism.

Previously characterized serum PTH heterogeneity includes the intact PTH (1-84) active peptide hormone, and inactive C-terminal fragments with N-termini between PTH (34-43). Thus, strategies for immunoassay evaluation of intact PTH concentrations in serum involved a solid phase antibody present in excess to overcome interfering C-terminal fragments and thus detecting intact PTH only using a N-terminally directed reporter antibody.

N-Terminal truncations yielding PTH species larger than PTH 34-84 have been isolated from human parathyroid cell monolayer.C-terminal fragments have been detected in extracts of parathyroid gland, and parathyroid effluent samples collected from hypercalcemic patients with hyperparathyroidism. Based on immunoassay value differences observed by our group and PTH immunoreactivity by Brossard et.al. (JCEM 81(11): 3923-3929) there appears to be differences in immunoassay values caused by fragment recognition in some commercially available intact PTH assays.

Evidence for a circulating inhibitor to PTH has arisen from studies of patients with pseudohyperparathyroidism. Despite elevated levels of immunoreactive PTH, bioactive PTH was normal when tested in bioassay systems. Plasma from these patients has been shown to diminish the biological activity of exogenous PTH in these *in vitro* bioassays.

To determine what PTH molecular forms circulate, and elucidate possible biological activity, a detection system was developed to elucidate the concentrations of intact PTH and the novel large molecular weight fragments of PTH, without interference or recognition of the hepatically generated C-terminal fragments. Data generated was evaluated for diagnostic potential with regard to primary hyperparathyroidism, and correlation to serum calcium in primary and uremic hyperparathyroidism.

JJC

Abstract

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

JWC

PTH Values inUremic Hyperparathyroidism

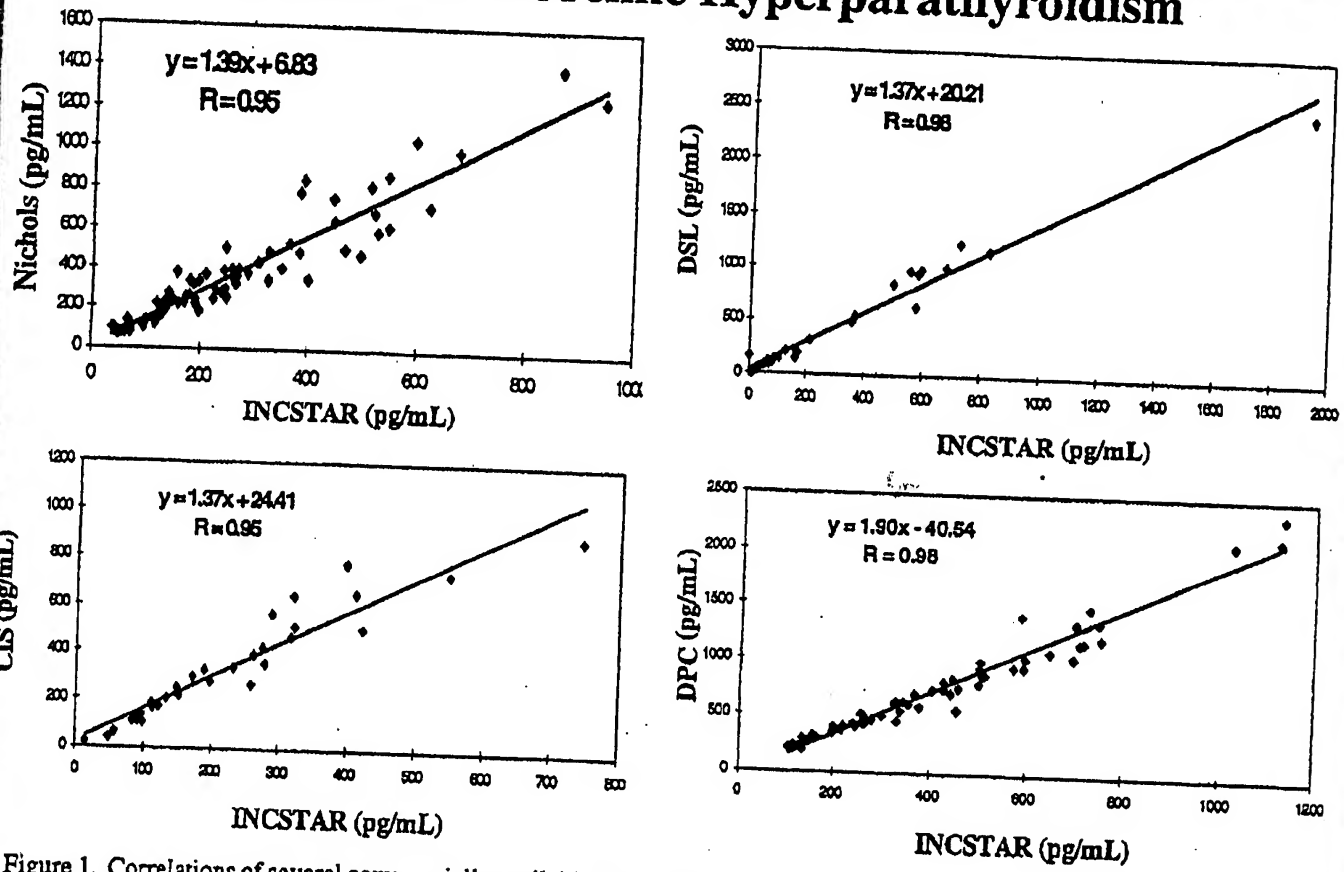


Figure 1. Correlations of several commercially available intact PTH assays verses the INCSTAR N-tact PTH SP intact PTH assay. All of the commercial assays evaluated indicate much greater cross-reactivity to the novel PTH fragments.

IMU-3285

INCSTAR PTH Values vs. Nichols PTH Values

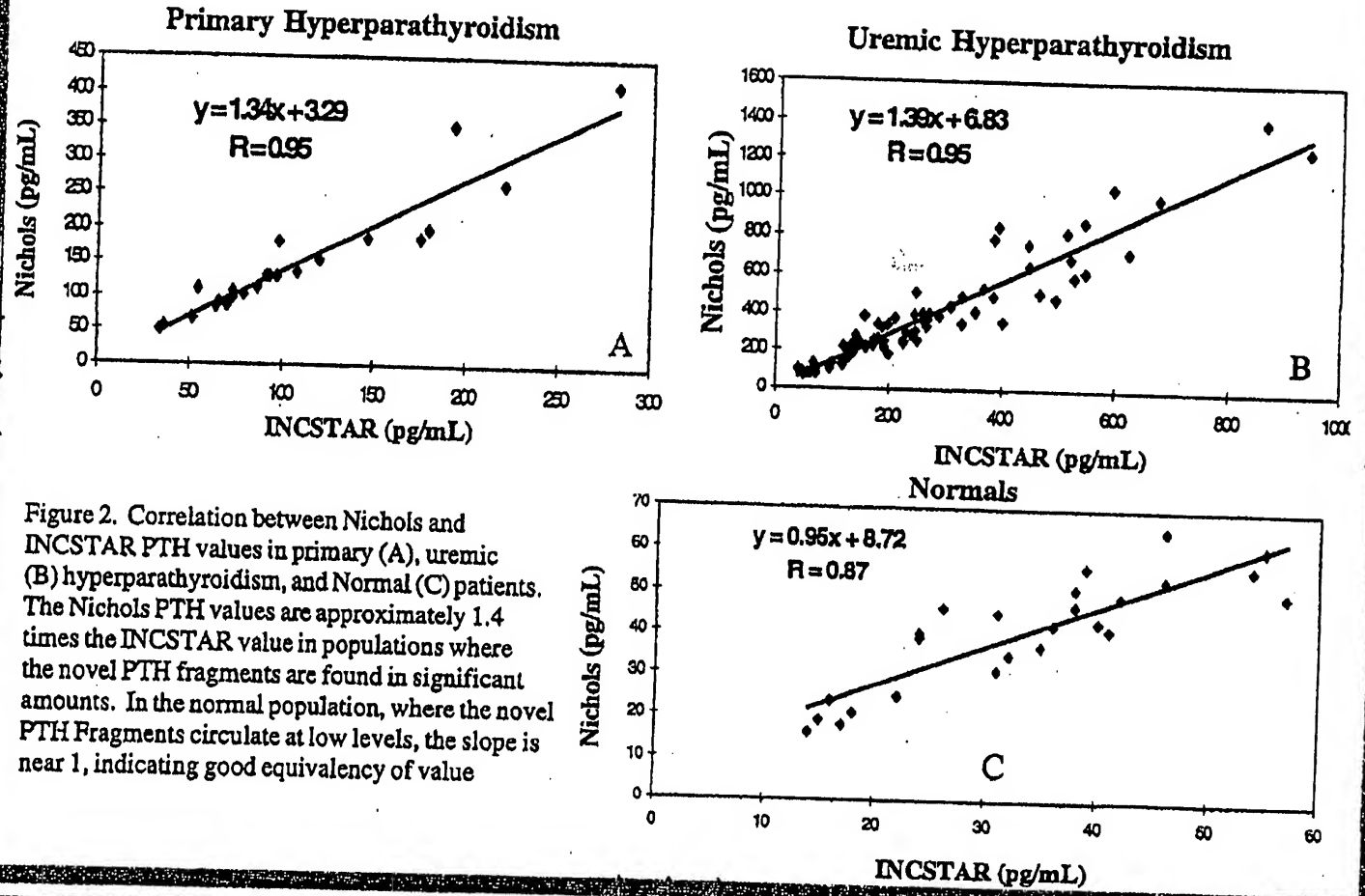
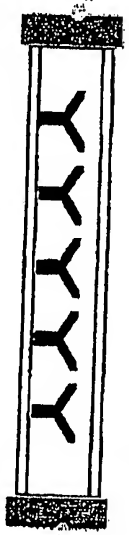


Figure 2. Correlation between Nichols and INCSTAR PTH values in primary (A), uremic (B) hyperparathyroidism, and Normal (C) patients. The Nichols PTH values are approximately 1.4 times the INCSTAR value in populations where the novel PTH fragments are found in significant amounts. In the normal population, where the novel PTH Fragments circulate at low levels, the slope is near 1, indicating good equivalency of value

Method of Isolation of PTH Molecular Forms



Delipidated EDTA plasma is loaded on the column. PTH Molecular forms containing all or parts of the (39-84) region are captured. The specifically bound protein is eluted with 0.2 M glycine pH 2.5

Anti-PTH(39-84) Immunoextraction Column

The protein eluted from the immunoextraction step is loaded onto a C_{18} reverse-phase HPLC column. The column resolves homologous proteins by size. 2-60% - 0.1% TFA/Acetonitrile : 0.1% TFA/dH20 over 58 minutes 1%/minute.

C_{18} Reverse Phase HPLC Column

HPLC Fractions were tested for PTH immunoreactivity

Figure 3. Method of isolation of PTH molecular forms.

IMU-3287

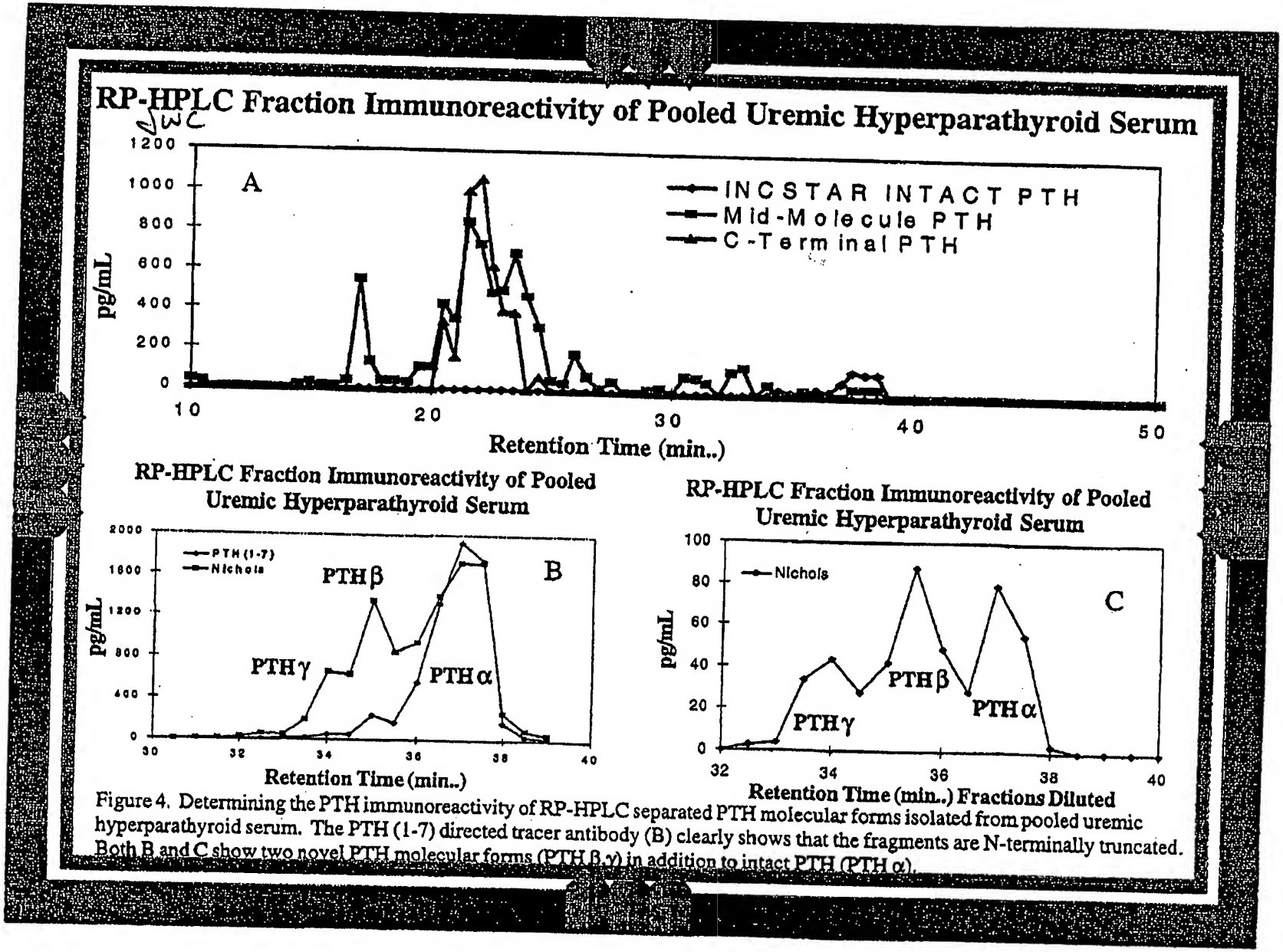


Figure 4. Determining the PTH immunoreactivity of RP-HPLC separated PTH molecular forms isolated from pooled uremic hyperparathyroid serum. The PTH (1-7) directed tracer antibody (B) clearly shows that the fragments are N-terminally truncated. Both B and C show two novel PTH molecular forms (PTH β,γ) in addition to intact PTH (PTH α).

IMU-3288

JWC

PTH Molecular Forms

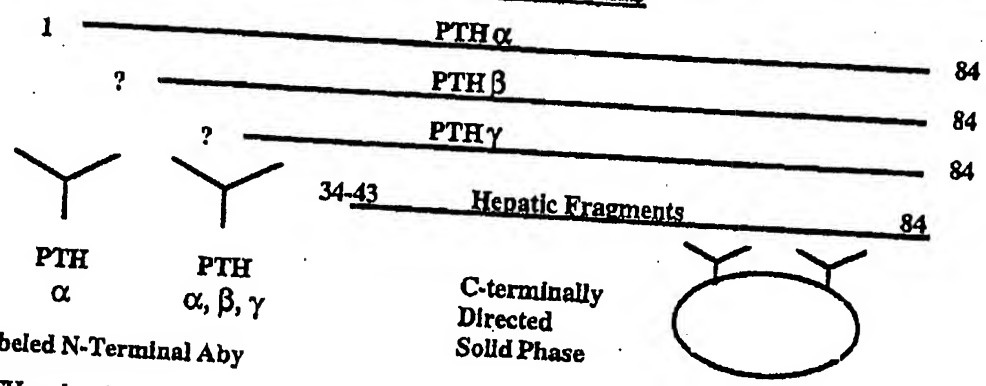


Figure 5. Defining PTH molecular forms isolated by RP-HPLC and the detection system used to estimate their concentration. These fragments have N-termini that extend beyond amino acid 34, and do not include hepatically generated fragments.

PTH Ratio

$$\frac{[\text{PTH } \alpha, \beta, \gamma] - [\text{PTH } \alpha]}{[\text{PTH } \alpha]} = \frac{\text{PTH } \beta,\gamma}{\text{PTH } \alpha}$$

This calculation yields a ratio of the two novel C-terminal Fragments / Intact PTH

The hepatically generated C-terminal fragments are NOT evaluated in this system

Figure 6. Defining the PTH Ratio to be the concentrations of the two novel PTH fragments to the concentration of intact PTH

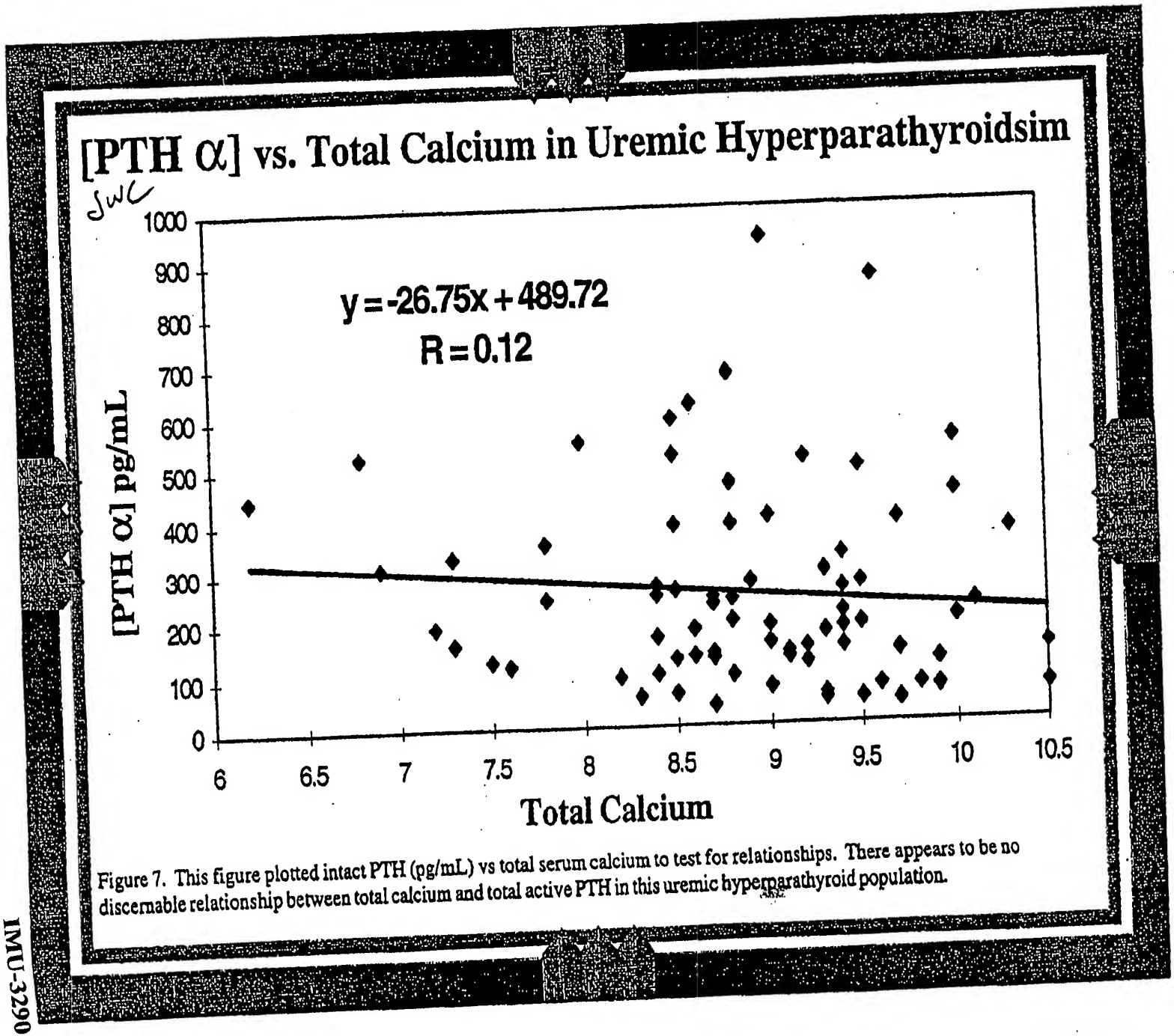


Figure 7. This figure plotted intact PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernable relationship between total calcium and total active PTH in this uremic hyperparathyroid population.

IMU-3290

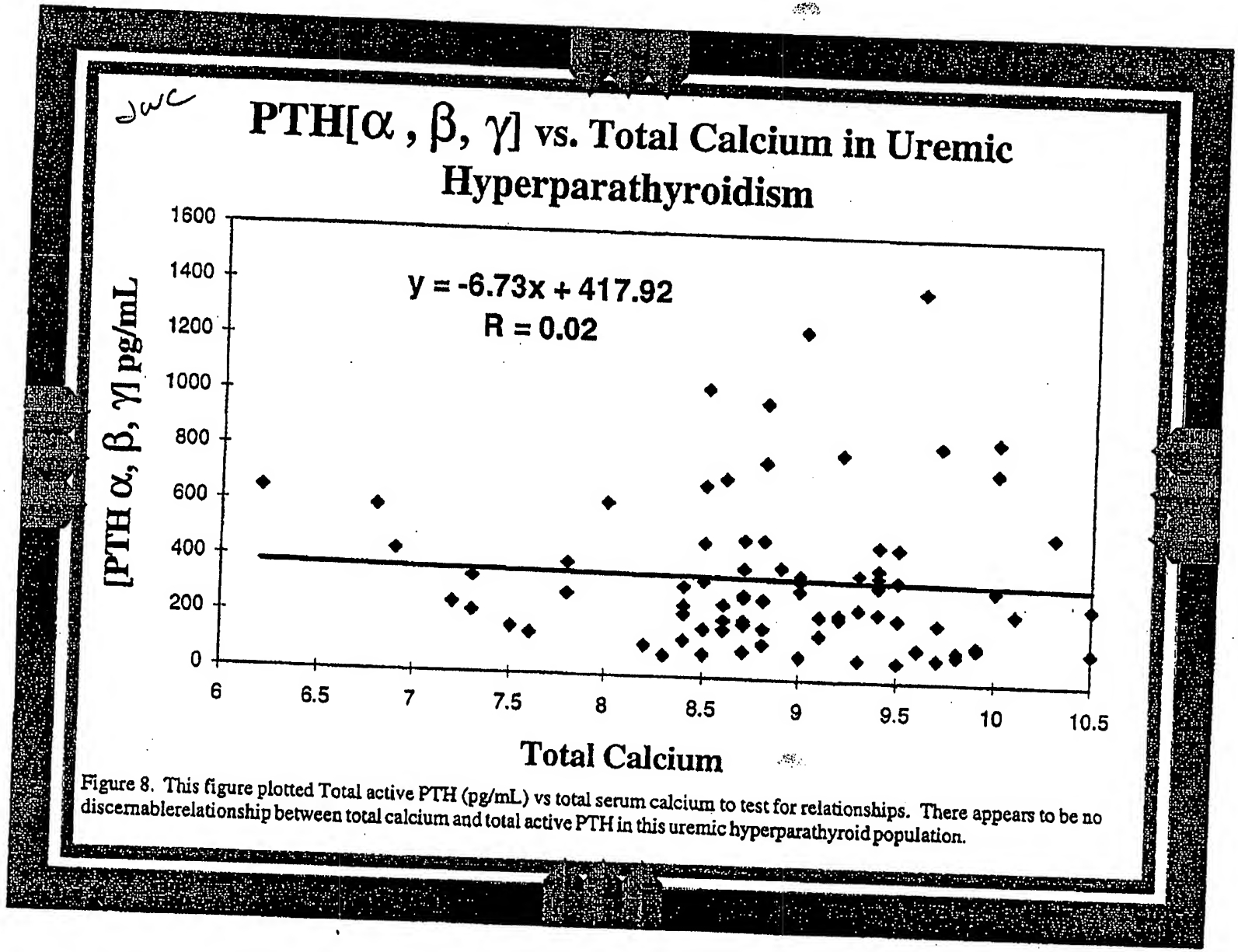


Figure 8. This figure plotted Total active PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernablerelationship between total calcium and total active PTH in this uremic hyperparathyroid population.

IMU-3291

PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Uremic Hyperparathyroidism

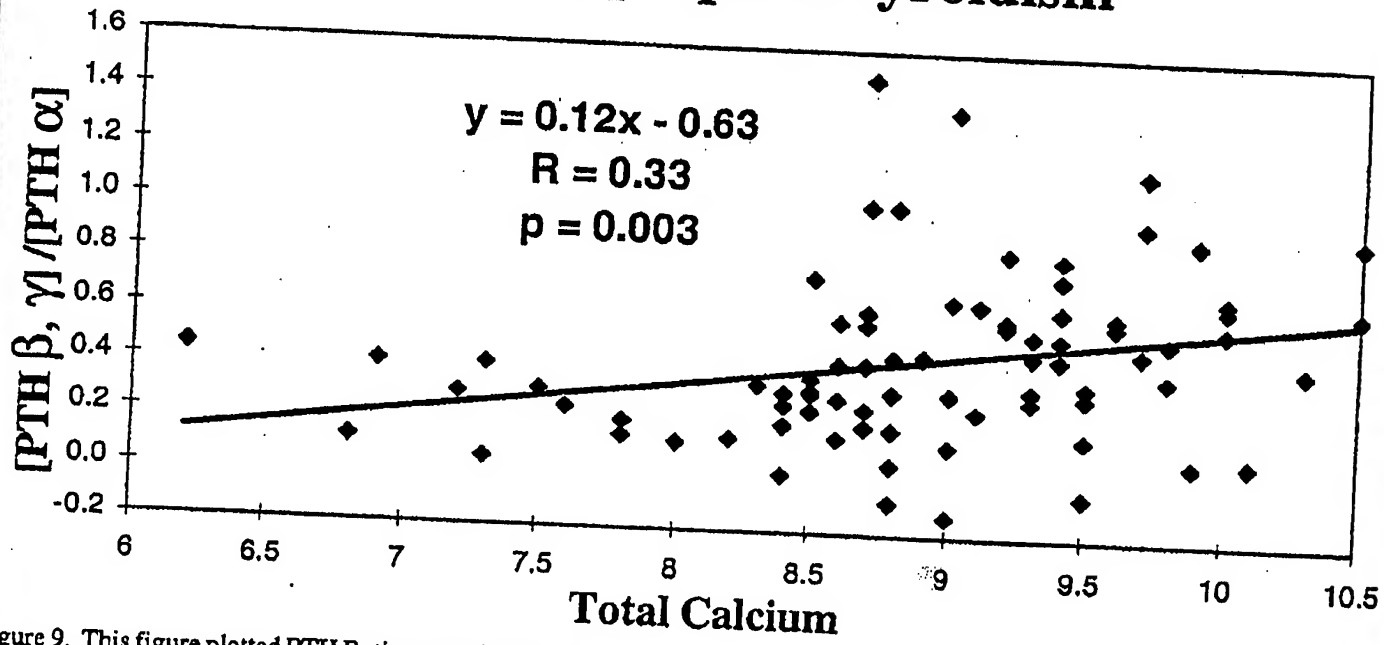


Figure 9. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, the corresponding serum calcium approaches normalization in uremic hyperparathyroidism even though most of these samples have elevated intact PTH levels. This trend is opposite to that in primary hyperparathyroidism. Endogenous and exogenous factors, such as calcitriol therapy, complicate calcium homeostasis in uremic hyperparathyroidism, and thus cause and effect conclusions cannot be made in this population without more information. However, this is consistent with an inhibitory effect of these fragments to PTH in these patients.

IMU-3292

JWC PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Primary Hyperparathyroidism

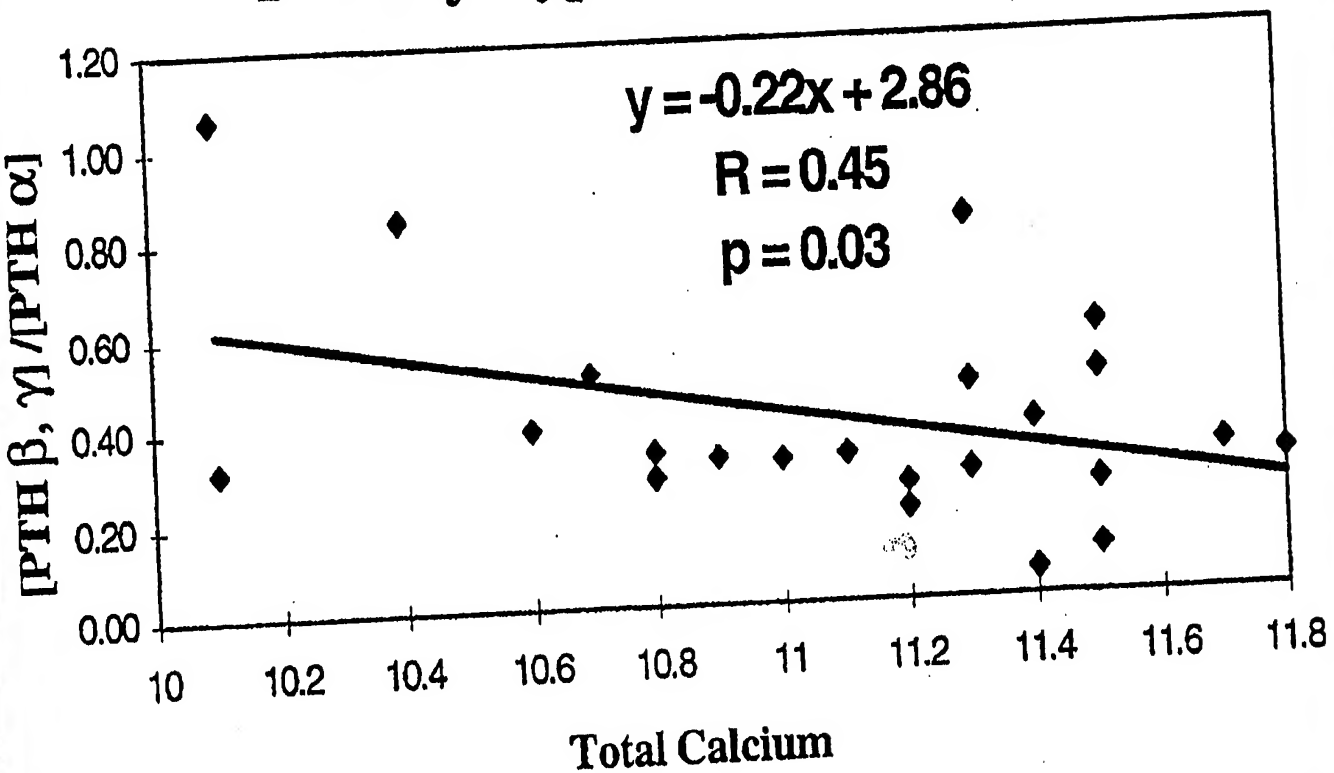


Figure 10. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, there is a trend of normalization of serum calcium in primary hyperparathyroidism even though all of these samples have elevated intact PTH levels. This is consistant with an inhibitory effect of these fragments to PTH action in this population.

IMU-3293

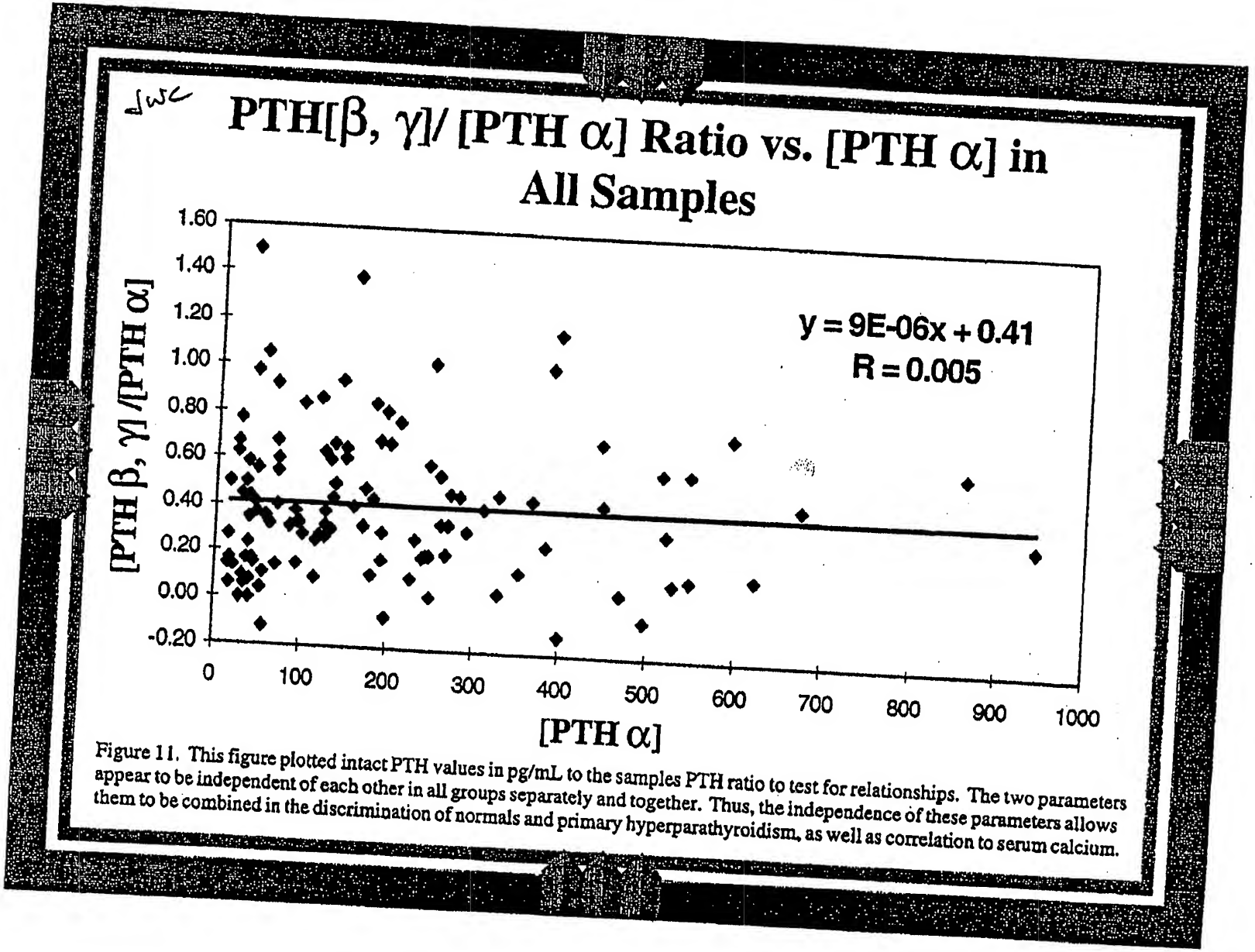
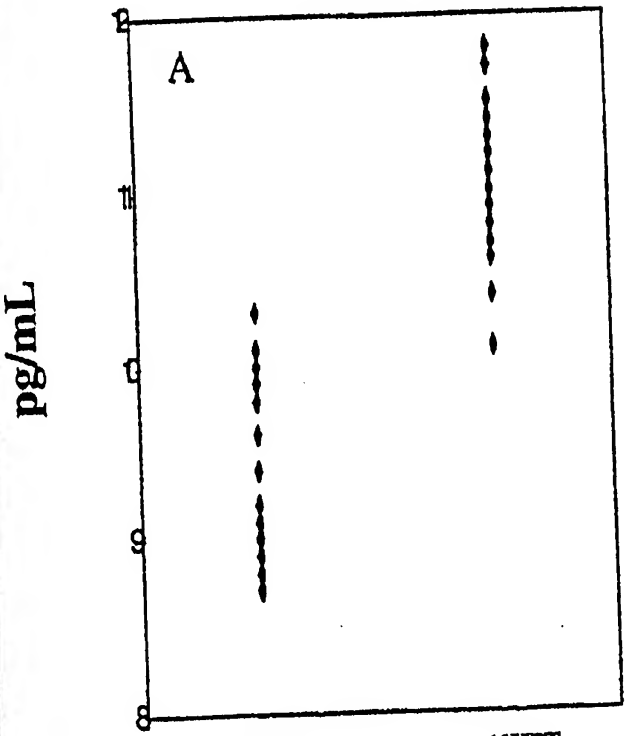


Figure 11. This figure plotted intact PTH values in pg/mL to the samples PTH ratio to test for relationships. The two parameters appear to be independent of each other in all groups separately and together. Thus, the independence of these parameters allows them to be combined in the discrimination of normals and primary hyperparathyroidism, as well as correlation to serum calcium.

JWC

Discrimination Power of Total Calcium



Discrimination Power of PTH

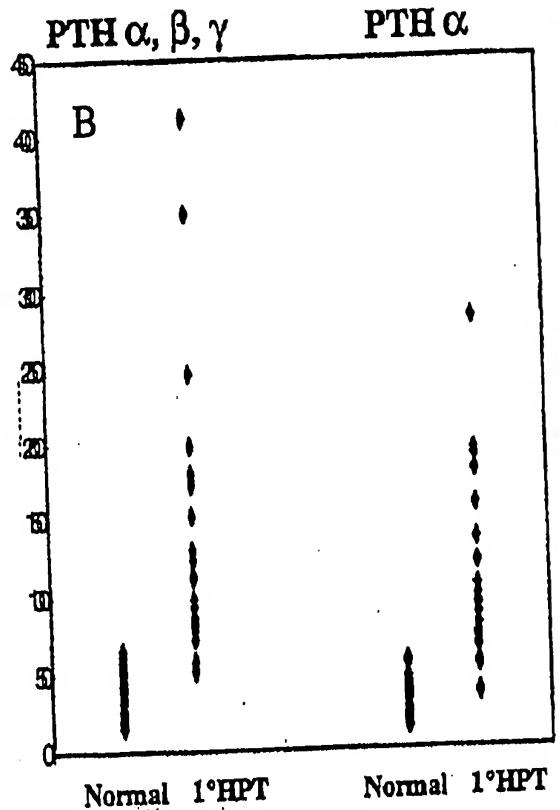


Figure 12. This figure used serum total calcium (A) and PTH values in pg/mL(B) to compare the discrimination power of calcium and PTH measurement in the separation of a normal and primary hyperparathyroid population. There is clearly significan overlap of the populations using both calcium and PTH determinations separately. These parameters are typically combined in a clinical situation to provide optimal discrimination, but which PTH parameter is the best is evaluated in Figure 13.

IMU-3295

Discrimination Power of PTH measurements + Total Calcium

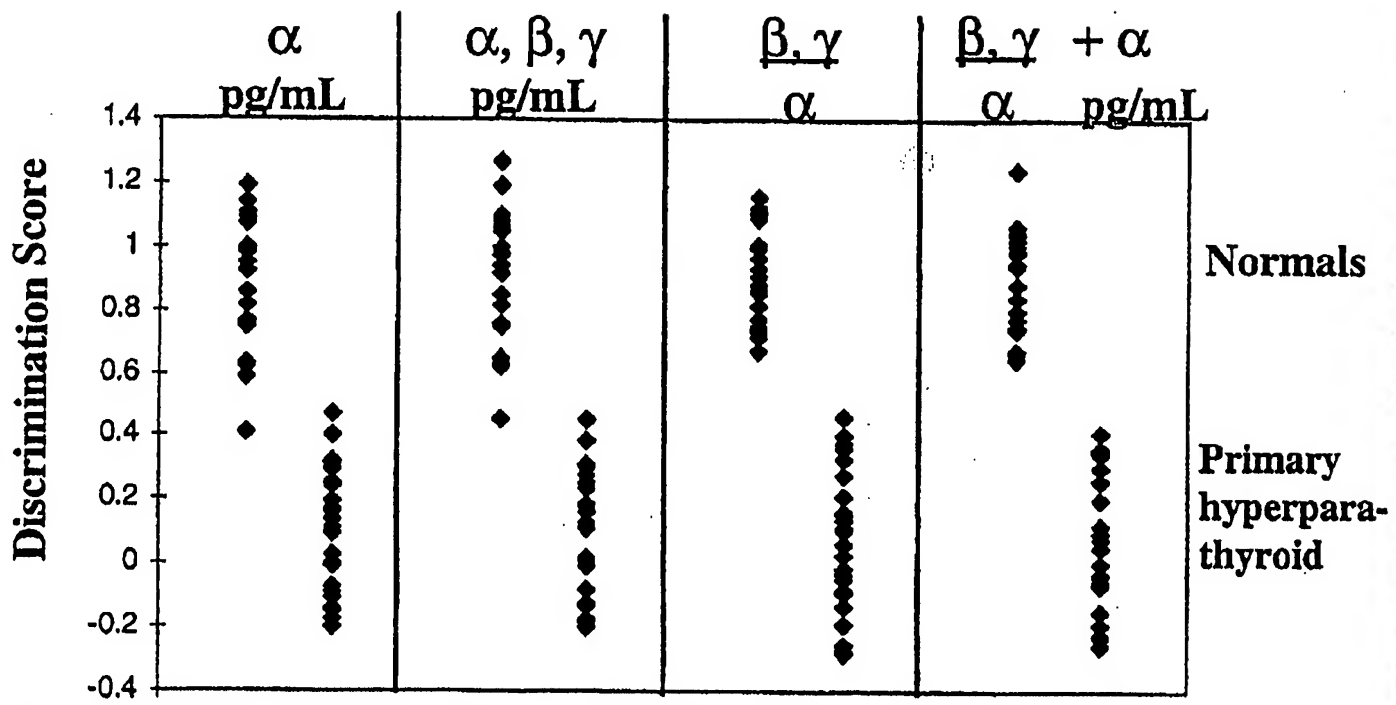


Figure 13. This figure used regression analysis to compare the discrimination power of a PTH measurement in addition to a total calcium determination in the separation of a normal and primary hyperparathyroid population. The combination of a PTH measurement with a serum calcium determination is the common clinical situation in diagnosis of primary hyperparathyroidism. The PTH ratio allows clear distinction of borderline cases into the respective populations.

IMU-3296

CONCLUSIONS

- There appears to be PTH heterogeneity found in circulation in patients with primary and uremic hyperparathyroidism beyond what has been characterized thus far.
- These novel PTH fragments appear to be N-terminally truncated and are reported in some commercially available intact PTH assays.
- As levels of the fragments approach those of Intact PTH, serum calcium trends toward normalization in patients with primary and uremic hyperparathyroidism
- Based on the data and references, we speculate that these fragments are a strong candidate for being a competitive inhibitor to intact parathyroid hormone action at the PTH/PTHrP receptor in patients with primary hyperparathyroidismand mediate its biological activity.
- Complete characterization of PTH molecular forms provides a better correlation to serum calcium, and provides greater clinical discrimination between primary hyperparathyroid and normals.

COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTHSP AND NICHOLS INTACT PTH ASSAYS.

Todd Jensen, Jon Spring, and John Colford (INCSTAR Corporation, Stillwater, MN 55082)

EXHIBIT 3

ABSTRACT

The N-terminus of the PTH molecule is required for PTH receptor interaction. It has been shown that cleavage of three amino acids from the N-terminus will inactivate PTH activity *in vivo*. Specificity is an advantage of a two antibody assay format of an IRMA. An N-terminal truncated circulating inhibitor to PTH has been hypothesized, but not found. Both the INCSTAR Intact PTHSP™ assay and the Nichols Allegro™ Intact PTH claim to be specific for the intact molecule. In a comparison between the two kits, patients with renal failure were assayed. The results showed that the Nichols assay results to be an average of 1.52 times the INCSTAR assay value (n = 14). Intact 1-84 PTH was spiked into clarified human serum and assayed in both kits. The results showed that the Nichols assay results to be an average of 1.07 times the INCSTAR assay value (n = 12).

Both assay tracers are directed to the N-terminal region of the intact PTH molecule. To compare specificity of the two assay tracers, we used a (7-84) C-terminal fragment. While not a defined byproduct of PTH metabolism *in vivo*, it could provide insight to the degree the population of antibodies in the tracer are directed to the N-terminus. Based on weight, target levels of 1500, 1000, and 500 pg/ml (7-84) PTH C-terminal fragment were spiked into clarified human serum. the Nichols assay had values of 520, 374, and 245 pg/ml, respectively. The INCSTAR assay had values of 220, 167, and 114 pg/ml, respectively. This showed that the Nichols results to be an average of 2.25 times the INCSTAR assay value (n = 14).

The INCSTAR tracer was substituted into the Nichols assay and the (7-84) PTH spiked serum samples were assayed, with the hybrid assay reporting 258, 191, and 121 pg/ml, respectively. This suggests that the INCSTAR tracer gives the INCSTAR assay greater specificity for the intact PTH molecule than the Nichols assay. These results also suggest the presence of a C-terminal fragment of PTH in renal samples that has not been currently identified.

INTRODUCTION

Recently, it has been reported that there is a non-(1-84) molecular form of PTH found in normals and renal samples that is immunoreactive in the Nichols Allegro™ Intact PTH assay (Brossard et al, Proc. Int. Cong. Endo, Vol2, OR60-5). The PTH molecule is degraded in the parathyroid gland itself and also in the liver. The breakdown products don't have most of the N-terminal 27 amino acids and lack hypercalcemic, hypocalciuric, and phosphaturic activity. These fragments have a considerable half-life and accumulate in circulation up to 20 times the concentration of the intact hormone. Their presence makes the immunologic assessment of the biologically active hormone difficult.

The hepatic fragments are generated when enzymes on the surface of Kupffer cells cleave the hormone in its 34-43 region. These fragments are formed at a rate dependent largely on the concentration of intact PTH. The N-terminal fragment is at very low concentrations in serum, suggesting a quick and thorough removal from circulation. Fragments generated in the parathyroid gland are truncated at both ends of the molecule. These fragments are not currently defined. In hypercalcemic patients of a non-parathyroid origin, the negative feedback mechanism will suppress PTH release to almost nothing, but the parathyroid gland will continue to release truncated fragments. In patients that have nonparathyroid forms of hypercalcemia, these fragments have been detected by a carboxy- terminal RIA but the N-terminus was not defined. All PTH fragments are removed from circulation by the kidney. In cases of renal insufficiency, fragments accumulate.

There appear to be differences in the values for renal samples among the Nichols Allegro™, DSL Active™, and INCSTAR N-tact® PTHSP assays. To explore these differences, PTH (1-84) and (7-84) immunoreactivity was validated between the assays. If PTH (1-84) equivalence is shown, renal sample differences will be significant. The results will be compared to reported non-(1-84) PTH immunoreactivity.

Dilution Linearity Validation

Pat. ID#	46	46 1:2	46 1:4
Nichols	1142	573	277
DSL	1233	707	378
INCSTAR	725	354	159
EXP(1-7)	860	383	158
EXP(7-84)	680	343	158

% Recovery	
46 1:2	46 1:4
100	97
115	123
98	88
89	74
101	93

Pat. ID#	48	48 1:2
Nichols	877	485
DSL	983	546
INCSTAR	593	278
EXP(1-7)	613	300
EXP(7-84)	533	253

% Recovery
48 1:2
111
111
94
98
95

Pat. ID#	49	49 1:2
Nichols	143	67
DSL	143	69
INCSTAR	107	51
EXP(1-7)	102	44
EXP(7-84)	104	59

% Recovery
49 1:2
94
96
95
87
112

PTH(7-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(7-84)
1	272	217	193	238
2	515	395	296	393
3	1262	1168	816	1094
4	1720	1623	1059	1693

$p<$ 0.0004

INCSTAR Tracer is unique
Nichols, DSL, and EXP (7-84)
are not shown different

PTH(1-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)
1	175	168	175	201	182
2	188	184	222	263	218
3	696	772	620	648	615
4	1058	1152	1045	1074	988

$p=$ 0.45

No Observed Difference

PTH Tracer Specificity.

	NSB	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	20	5419	40	1452	3643	3294	8	0	12	14	19
DSL	559	11627	415	5585	8922	6916	556	364	424	319	278
Nichols	195	2982	139	968	2606	2136	153	138	139	220	142

Yes is a signal 2 SD above Background

	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No
DSL	Yes	No	Yes	Yes	Yes	No	No	No	No	No
Nichols	Yes	No	Yes	Yes	Yes	No	No	No	No	No

Values from two separate assays confirming differences in (7-84) Spiked serum values between Nichols and INCSTAR

FRAGMENT(7-84) SPIKES (pg/mL)

	Target Value*	INCSTAR Assay	Nichols Assay	DIFFERENCE
Assay 1	1500	511	774	-262
	1000	366	577	-211
	500	220	346	-125
Assay 2	1000	220	520	-300
	400	167	374	-207
	250	114	245	-131

* - Value based on assumption of 100% purity and quantitative transfer in dilution

MEAN=	-206.0	
Variance=	4835.4	
Standard Error=	28.39	
***t*=**	7.26	
***p*=**	0.00078	Significant difference

Comparison of (1-84) Spiked serum values and controls between Nichols and INCSTAR

		INTACT (1-84) (pg/mL)		
		INCSTAR	Nichols	DIFFERENCE
Serum Spikes	**spike 1**	2805	2883	-77
	spike 2	1617	1712	-95
	spike 1 Diluted 1:10	273	236	37
	spike 2 Diluted 1:10	157	152	5
	spike 3	1656	1589	67
	spike 4	1740	1724	16
	spike 3 Diluted 1:10	183	212	-29
	spike 4 Diluted 1:10	164	190	-26
Kit Controls	**NJ**	36	31	6
	NK	265	214	51
	(INC)L1	33	34	-1
	(INC)L2	415	330	85
	(INC)L1	49	41	8
	(INC)L2	447	426	21

MEAN of Ln*=	0.0456
Standard Error of Ln*=	0.0330
t=*	1.38
p=*	0.1896

NOT a significant difference

* - Data generated from the Natural Log transformed data. This transformation is necessary due to the large range the data. It reduces the chances for error.

Patient Sample Comparison

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)	
1	196	197	170	195	163	renal failure
2	17	8	15	20	14	kidney stones
3	920	943	580	568	478	renal failure/hyperPTH
4	176	144	161	220	148	n/a
5	17	11	12	15	15	renal failure
6	375	540	366	542	341	renal failure
7	66	75	51	60	60	renal failure
8	28	25	26	26		renal failure
9	50	59	35	34	38	renal failure
10	89	104	80	79	75	renal failure
11	1043	1160	825	988	831	renal failure
12		0	6			renal failure
13	166	209	126	126	117	bone disease
14	78	99	69	115	68	renal failure
15	31	35	28	32	31	renal failure
16	17	11	17			kidney stones
17	930	959	552	588	473	renal failure/hyperPTH
18	202	192	166	174	172	renal failure
20	143	159	0	141	92	renal failure
21	121	120	69	63	72	renal failure
22	773	850	498	604	523	renal failure/hyperPTH
23	34	27	24	28	23	renal failure
24	104	105	84	85	79	renal failure
25	11	7	12	12	11	renal failure
26	293	316	211	194	186	renal failure
27	105	135	87	81	86	renal failure
28	473	495	353	380	370	renal failure
29	28	22		27	24	n/a
30	74	94	71	70	62	renal failure
31	69	88	59	48	55	renal failure
32	892	1000	677	787	651	renal failure/hyperPTH
33	14	6	13		18	renal failure
34	648	629	575	526	544	renal failure
35	2043	2476	1942	2230	1904	renal failure/hyperPTH
46	1142	1233	725	860	680	renal failure/hyperPTH
47	31	29	31		31	kidney stones
48	877	983	593	613	533	renal failure/hyperPTH
49	143	143	107	102	104	renal failure
50	49	42	33	31	35	renal failure

Significantly Different Groups $p<$ 0.0001

There are two sharp, well separated groups, by Tukey's test

Group 1
Nichols
DSL

Group 2
INCSTAR
EXP(1-7)
EXP(7-84)

CONCLUSIONS

In the Nichols Allegro™ Intact PTH, DSL Active™ PTH and INCSTAR N-tact® PTHSP equivalence of values for samples with clearly defined 1-84 was validated using spikes throughout the standard range, and dilutions to verify values. There is no significant difference for PTH(1-84) values between the three kits ($p = 0.45$). Values for renal samples show a significant difference between the INCSTAR Assay and the other two kits (Nichols and DSL) which report up to twice the INCSTAR value ($p < 0.0001$). PTH (7-84) immunoreactivity was the same for the Nichols, DSL, and one experimental INCSTAR tracer. Two differently purified tracers from INCSTAR with significantly different PTH(7-84) immunoreactivity did not report different values for renal samples, so PTH(7-84) immunoreactivity itself does not elucidate non-(1-84) PTH immunoreactivity. These results suggest that the differences between INCSTAR assay values and the other two kits (Nichols and DSL) is the differing levels of cross-reactivity to fragments present in renal samples, likely of parathyroid gland origin. Based on specificities of each tracer, the N-termini of the fragment(s) reside inside the PTH (7-28) region, and likely end in the PTH (13-28) region. If these data are confirmed in sequencing the fragment, it would not have PTH-receptor mediated biological activity.

Materials and Methods

Assaying of Spiked samples and patient samples:

Respective assay protocols were followed for the collection of data. The PTH(1-84) and PTH(7-84) were purchased from BACHEM. The serum matrix used to spike into was low pH stripped, charcoal stripped, defibrinated, delipidated normal serum. The peptides were dissolved in 5% acetic acid, and serially diluted in the serum matrix to target values within each assays standard range. The spikes were targeted to values based on weight on the vial label, and the entire contents were dissolved with no quantitative assessment of a concentration. All samples were assayed side by side in each kit. The neat value for the serum matrix without spiking was 0.0 pg/mL in both assays. Both Nichols and INCSTAR kit controls were assayed in each others kits and reported as (1-84) spikes.

Peptide Coated Wells for the Specificity Screen:

(modified from Ball et. al., J. Imm. Meth. 171(1994) 37-44)
Briefly: Poly Lys:Tyr (1:1) is coated in PBS to microtiter plates, washed, then the Lys amines are activated with glutaraldehyde. The plates are washed again, and peptides are added to specific wells and left to conjugate over night. The Shiff bases are reduced to primary amines with sodium cyanoborohydride, then the wells are blocked to prevent NSB.

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Development of a Novel Immunoradiometric Assay Exclusively for Biologically Active Whole Parathyroid Hormone 1-84: Implications for Improvement of Accurate Assessment of Parathyroid Function

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ABSTRACT

We developed a novel immunoradiometric assay (IRMA; whole parathyroid hormone [PTH] IRMA) for PTH, which specifically measures biologically active whole PTH(1-84). The assay is based on a solid phase coated with anti-PTH(39-84) antibody, a tracer of ^{125}I-labeled antibody with a unique specificity to the first N-terminal amino acid of PTH(1-84), and calibrators of diluted synthetic PTH(1-84). In contrast to the Nichols intact PTH IRMA, this new assay does not detect PTH(7-84) fragments and only detects one immunoreactive peak in chromatographically fractionated patient samples. The assay was shown to have an analytical sensitivity of 1.0 pg/ml with a linear measurement range up to 2300 pg/ml. With this assay, we further identified that the previously described non-(1-84)PTH fragments are aminoterminally truncated with similar hydrophobicity as PTH(7-84), and these PTH fragments are present not only in patients with secondary hyperparathyroidism (2°-HPT) of uremia, but also in patients with primary hyperparathyroidism (1°-HPT) and normal persons. The plasma normal range of the whole PTH(1-84) was 7–36 pg/ml (mean ± SD: 22.7 ± 7.2 pg/ml, $n = 135$), whereas over 93.9% (155/165) of patients with 1°-HPT had whole PTH(1-84) values above the normal cut-off. The percentage of biologically active whole PTH(1-84) (pB%) in the pool of total immunoreactive "intact" PTH is higher in the normal population (median: 67.3%; SD: 15.8%; $n = 56$) than in uremic patients (median:53.8%; SD: 15.5%; $n = 318$; $p < 0.001$), although the whole PTH(1-84) values from uremic patients displayed a more significant heterogeneous distribution when compared with that of 1°-HPT patients and normals. Moreover, the pB% displayed a nearly Gaussian distribution pattern from 20% to over 90% in patients with either 1°-HPT or uremia. The specificity of this newly developed whole PTH(1-84) IRMA is the assurance, for the first time, of being able to measure only the biologically active whole PTH(1-84) without cross-reaction to the high concentrations of the aminoterminally truncated PTH fragments found in both normal subjects and patients. Because of the significant variations of pB% in patients, it is necessary to use the whole PTH assay to determine biologically active PTH levels clinically and, thus, to avoid overestimating the concentration of the true biologically active hormone. This new assay could provide a more meaningful standardization of future PTH measurements with improved accuracy in the clinical assessment of parathyroid function. (J Bone Miner Res 2001;16:605–614)

Key words: parathyroid hormone, immunoassay, hyperparathyroidism, uremia, parathyroid hormone fragment

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INTRODUCTION

THE INVENTION and evolution of immunoassays measuring human parathyroid hormone (PTH; parathyrin) has provided us with a better understanding of the biological and biochemical nature of this polypeptide hormone and a better tool for the clinical diagnosis and monitoring of the diseases related to primary hyperparathyroidism (1°-HPT), secondary hyperparathyroidism (2°-HPT), and hypoparathyroidism.[1–5] Circulating PTH is immunochemically heterogeneous and the midregional/C-terminal PTH fragments are known to be significantly accumulated in some disease conditions, for example, chronic renal failure.[6] Prior competitive immunoassays for PTH detect a mixture of different PTH fragments as well as the whole biologically active PTH(1-84); hence, these assays have not accurately assessed the level of circulating biologically active hormone and the function of the parathyroid glands. Because the whole or complete molecule of PTH(1-84) is the major circulating form of the serum biologically active hormone, which is capable of binding and activating the PTH-1 receptor on kidney and bone, the primary goal of developing and using intact PTH sandwich assays was to measure biologically active PTH(1-84) exclusively.[3]

Since 1987, commercially available "intact" PTH assays have greatly increased assay sensitivity and simplified the assay procedures for PTH measurement. However, the clinical use of these intact PTH assays is still fraught with challenges. For example, intact PTH levels frequently overestimate the presence and severity of parathyroid-mediated osseous abnormalities in uremic patients.[7–9] In addition, interlaboratory discordances of PTH values arose when different intact PTH kits from different manufacturers were used. One of the explanations could be that different paired antibodies with different specificities are used to form the sandwich assay for intact PTH. Indeed, recent studies have revealed that there are circulating non-(1-84) PTH fragments that interfere significantly with intact PTH measurements obtained from commercial assays in uremic patients.[10,11] One of these studies using high-performance liquid chromatography (HPLC) and different intact PTH assays has found that more than 30% of total immunoreactive intact PTH is comprised of non-(1-84) PTH fragments in this group of patients. Therefore, those intact PTH assays are not truly intact specific and still measure a mixture of the biologically active whole PTH(1-84) and large PTH fragments that show similar hydrophobicity as synthetic PTH-(7-84).[10]

It is our opinion that an optimal immunoassay for PTH should measure only the clinically significant, biologically active form of PTH, which is capable of binding to the G protein–linked PTH receptors,[12,13] which initiates signal transductions in the intracellular biochemical process resulting in the regulation of calcium metabolism. In addition to its specificity,[14,15] this optimal PTH assay should be sensitive, to allow diagnosis of hyperparathyroidism[16,17]; easy to perform; and of high performance in assay characteristics. To meet these goals for assaying PTH, we developed a whole PTH(1-84) immunoradiometric assay (IRMA) using a PTH(39-84) region–specific polyclonal capture antibody and a PTH(1-4) highly specific polyclonal label antibody. With these antibodies, this assay is restricted to measure only the authentic whole PTH(1-84) without any cross-reaction with the high levels of non-(1-84) PTH fragments found in patient samples. Clinical studies have shown that this specific whole PTH(1-84) assay unexpectedly provides a unique tool for the diagnosis of patients with parathyroid diseases. In studies with this new whole PTH IRMA and HPLC fractionated clinical samples, we clearly show that previously described non-(1-84) PTH fragments are amino-terminally truncated polypeptides and these PTH fragments are significantly present not only in uremic patients but also in patients with 1°-HPT and normal persons. Moreover, we further show that the ratio of full-length PTH(1-84) to aminoterminally truncated PTH fragments is significantly variable from patient to patient with HPT.

MATERIALS AND METHODS

Chemicals and reagents

Most chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO, USA). Synthetic PTH(1-84) was from Peninsula Laboratories, Inc. (Belmont, CA, USA). Synthetic peptides of PTH(7-84), PTH(44-68), PTH(53-84), and PTH(39-84) were purchased from Bachem (Torrance, CA, USA). [Tyr34]PTH(1-34)amide {PTH(1-34)}, [Tyr34]PTH(2-34)amide {PTH(2-34)}, [Tyr34]PTH(3-34)amide {PTH(3-34)}, [Tyr34]PTH(4-34)amide {PTH(4-34)}, [Tyr34]PTH-(5-34)amide {PTH(5-34)}, and [Tyr34]PTHrP(1-34)amide {PTHrP(1–34)} fragments were synthesized by the Massachusetts General Hospital Polymer Core Facility (Boston, MA, USA). Cyanogen bromide–activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). One liter of 0.01 M phosphate-buffered saline (PBS; pH 7.4) contained 0.23 g sodium dihydrogen phosphate, 1.2 g disodium hydrogen phosphate, and 8.5 g sodium chloride. One liter 0.1 M glycine hydrochloride buffer (pH 2.5) contained 8.76 g sodium chloride. Assay wash buffer was 0.01 M PBS (pH 7.4) with 0.01% Triton X-100. Nichols intact PTH IRMA kit was purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA, USA).

Standards and controls for the whole PTH IRMA were prepared by adding synthetic PTH(1-84) to a normal human serum that did not show any detectable PTH level with the intact PTH assay. The concentrations of the standard set were 0, 10, 16, 46, 165, 700, and 2300 pg/ml. All standards and controls were aliquoted, lyophilized, and stored at 2–8°C.

Goat anti-PTH(39-84) polyclonal antibody coated onto 5/16-in polystyrene beads (Hoover Precision Products, Sault Ste. Marie, MI, USA) were used as the solid phase. The antibody was prepared by affinity purification. Briefly, synthetic PTH(39-84) peptide was conjugated covalently to Sepharose 4B gel using the manufacturer's suggested procedures by mixing the gel with the peptide at room temperature for 16 h. The peptide-bound Sepharose 4B gel was transferred to a chromatography column and the packed column was washed and equilibrated with 0.01 M PBS. Goat anti-PTH(39-84) antiserum was loaded onto the column. Unbound protein and other matrix components were

washed away using 0.01 M PBS and the specific goat anti-PTH(39-84) polyclonal antibody was eluted with 0.1 M glycine hydrochloride buffer. The eluted polyclonal antibody was neutralized and stored at 2–8°C. The purified goat anti-PTH(39-84) polyclonal antibody was attached physically onto the surface of the polystyrene beads by means of passive absorption.[5,18] The beads were blocked by Scancoat (Scantibodies Laboratory, Santee, CA, USA) and finally dried at room temperature. These antibody-coated beads were then stored at 2–8°C and were ready for assay use.

^{125}I-PTH(1-4) region–specific polyclonal antibody was used as the assay signal antibody. This antibody also was affinity-purified by the same procedure as described previously. The chloramine T method was used for the iodination of this most N-terminal PTH–specific antibody. A PD-10 column was used for the separation of the ^{125}I-labeled antibody from the free iodine. Selected fractions of labeled antibody were pooled and diluted using 0.01 M sodium phosphate–based buffer approximately to 300,000 disintegrations per minute (dpm) per 100 μl. This solution was the final tracer to be used in the whole PTH IRMA.

IRMA for whole PTH(1-84)

A single incubation step IRMA specific for the whole PTH(1-84) was developed and optimized with the previously mentioned assay reagents. Briefly, 200 μl of assay standards, controls, and patient samples were pipetted into appropriately labeled 12 mm × 75 mm polypropylene test tubes. One hundred microliters of ^{125}I-labeled PTH(1-4)–specific antibody tracer solution and one goat anti-PTH(39-84) polyclonal antibody–coated bead were added to all test tubes. The immunochemical reaction was conducted at room temperature with shaking at 170 rpm for 18–22 h. During this assay incubation period, the immunochemical reaction forming the sandwich of {solid-phase goat anti-PTH(39-84) antibody}–{whole PTH(1-84)}–{^{125}I-goat anti-PTH(1-4) antibody} takes place in correlation with the amount or concentration of whole PTH(1-84) in the test sample. All beads in the test tubes except the total count tube were washed with the wash solution, and the radioactive signals from each bead were counted for 1 minute using a gamma scintillation counter (ISO-Data, Palatine, IL, USA). The data were processed and calculated using nonlinear regression data reduction software.

Chromatographic separations

Sep-Pak Plus C_{18} cartridges (Waters Chromatographic Division, Milford, MA, USA) were used for the extraction of PTH from serum samples derived from single individuals or pools from up to 10 individuals among uremic patients, 1°-HPT patients, and normal persons. One cartridge was used for each 3 ml of serum and extracted volumes varied between 12 and 25 ml depending on the PTH concentration.[19] The eluted samples from the cartridges were first evaporated with nitrogen and then the residual volume was freeze-dried. All extracted samples were then reconstituted with 2 ml of 0.1% trifluoroacetic acid and chromatographed on a C_{18} μ-Bondapak analytical column (3.8 × 200 mm; Waters Chromatographic Division) using a noncontinuous linear gradient of acetonitrile (15–50% in 1.0 g/liter trifluoroacetic acid). After evaporation and freeze-drying, each 1.5-ml fraction was reconstituted to 1 ml with 0.7% bovine serum albumin (BSA) in H_2O. Both the whole PTH IRMA and the Nichols PTH IRMA were used to determine the PTH values in each fractionated sample. The recovery of intact PTH throughout all these procedures was 109 ± 10% in normal individuals, 70 ± 14% in renal failure patients, and 108 ± 4% in 1°-HPT.

Samples

One hundred and thirty-five normal human EDTA-plasma and serum samples were obtained from healthy laboratory staff members or donors, with an age ranging from 20 to 62 years (mean ± SD: 42 ± 12.6 years). Three hundred and eighteen patient samples of EDTA plasma (frozen/thawed once) were obtained from uremic patients with ongoing dialysis. The serum samples were collected and allowed to clot for approximately 30–40 minutes at room temperature and then centrifuged at 4°C. EDTA-plasma blood was collected into EDTA sample collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and immediately centrifuged at 4°C. The separated EDTA plasma and serum samples were stored at −20°C until used. One hundred and sixty-five samples (111 serum and 54 EDTA-plasma) from patients with surgically proven 1°-HPT were obtained from −70°C sample banks.

A stability study of whole PTH(1-84) in clinical samples was conducted with EDTA plasma, heparinized plasma, and serum. All three types of samples were drawn from three blood donors at the same time. One of the individuals was a patient with 1°-HPT, the other two were normal persons. Samples from only one of the normal persons, who had an original whole PTH(1-84) value of 9 pg/ml, were spiked with synthetic PTH(1-84) to an approximate level of 100 pg/ml. For this study the serum was obtained after routine blood clotting at room temperature for 30 minutes and centrifuged at 2–8°C for 10 minutes; for both EDTA-plasma and heparinized plasma the whole blood was placed immediately into an ice bath and centrifuged at 4°C. All samples were pooled, aliquoted at a 2-ml quantity, and incubated in 2-ml quantities at both room temperature and 2–8°C for 0–72 h, and frozen at −20°C until measured.

RESULTS

Performance characteristics of the whole PTH IRMA

Calibration curve and precision: An IRMA for whole PTH(1-84) was developed and optimized using the assay procedure described previously. A typical whole PTH IRMA standard curve is shown in Fig. 1. The affinity-purified antibodies used in the assay, either as capture antibody or as ^{125}I-labeled antibody, ensured the strong immunoreaction of antigen-antibody binding and low background of 526 ± 86 cpm (mean ± SD) for six iodinations. The intra-/interassay precision was determined by assaying

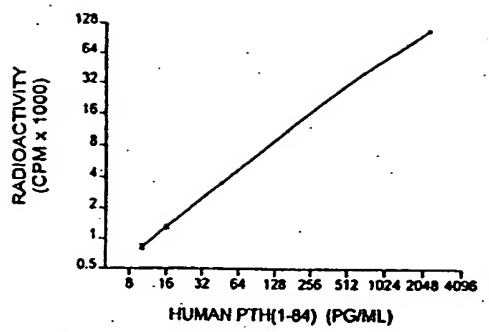
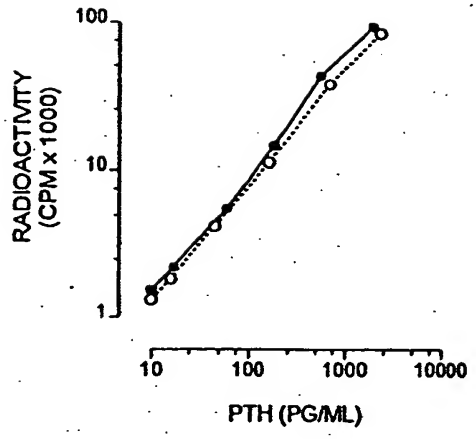


FIG. 1. A typical calibration curve obtained with the IRMA for whole PTH(1-84) as described in the Materials and Methods section. Data are expressed as means ± SD of triplicate measurements and are represented directly by the radioactivity (cpm × 1000).



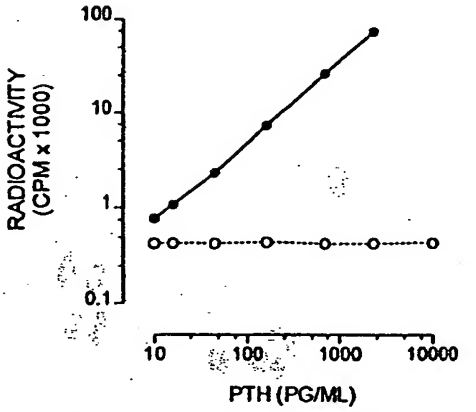


FIG. 2. Characterization of assay specificity for two PTH IRMAs [top, Nichols intact PTH IRMA; bottom, whole PTH IRMA; solid-circle, PTH(1-84); open-circle, PTH(7-84)].

two control samples with whole PTH(1-84) concentrations of 32 pg/ml and 340 pg/ml either by performing 60 replicate measurements in the same assay or in 40 different assays. The within-run variation was 6.1% and 2.3% and the between-run variation was 8.9% and 2.9%. No high-dose "hook" effect was observed after the addition to test samples of synthetic PTH(1-84) up to 20,000 pg/ml.

Analytical sensitivity: The assay detection limit was determined to be 1.0 pg/ml, which was the lowest measurable concentration of PTH value distinguishable from zero. It was determined by measuring the assay standard zero 22 times in the same assay and the value corresponding to the counts of 2 times of SD above the mean of the zero standard. This assay sensitivity was confirmed by validating with three independent production batches of the whole PTH reagents.

Linearity and analytical recovery: Three patient serum samples with PTH concentrations over 60 pg/ml were diluted 1:2, 1:4, and 1:8 with the assay zero standard. The percent recovery was determined after measurement of the diluted samples. Satisfactory assay linear recoveries of 93–112% were observed within the assay measurement range of 1.0–2300 pg/ml, respectively. Sample spiking recovery was determined by adding two different amounts of PTH into three patient serum samples with known whole PTH(1-84) values. The percentage of sample spike recovery was calculated following the assay of the spiked samples in comparison with the expected value. Recoveries from 99.3 to 113% were observed.

Analytical specificity and interference: Assay specificity to synthetic PTH(7-84) was studied by comparing this whole PTH IRMA with the Nichols intact PTH IRMA. Nearly 100% cross-reaction to this fragment was observed with the Nichols intact PTH assay, but no cross-reaction was detected with this newly developed whole PTH IRMA even at a PTH(7-84) concentration of 10,000 pg/ml (Fig. 2). The whole PTH IRMA also showed no cross-reaction to other PTH fragments, such as PTH(1-34), PTH(39-84), PTH(44-66), and PTH(53-84).

Evaluating the specificity of tracer antibodies

The specificities of the two ^{125}I-labeled antibodies from the Nichols intact PTH IRMA and this new whole PTH IRMA were compared. Calibrators with a constant PTH(1-84) concentration of approximately 440 pg/ml were determined by both assays with increasing amounts (from 0 to 100,000 pg/ml) of coincubated aminoterminal PTH analogues. In the Nichols intact PTH IRMA, specific binding of ^{125}I-labeled tracer antibody to PTH(1-84) was reduced progressively by increasing concentrations of PTH(1-34), PTH(2-34), PTH(3-34), PTH(4-34), and PTH(5-34). In the whole PTH IRMA, in contrast, the bound signal of ^{125}I-labeled antibody was only competitively inhibited by PTH(1-34). No binding reduction could be determined by increasing concentrations of PTH(2-34), PTH(3-34), PTH(4-34), and PTH(5-34) (Fig. 3). Increasing concentrations of PTHrP(1-34) had no inhibitory effect on the ^{125}I-labeled antibodies in both assays.

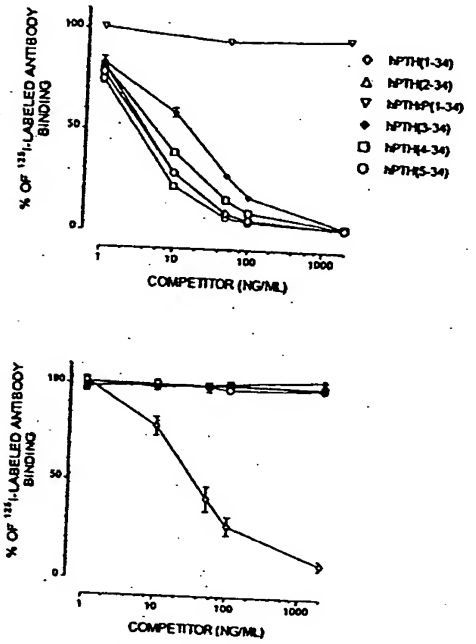


FIG. 3. Characterization of two tracer antibodies used in the Nichols intact PTH IRMA (top) and the whole PTH IRMA (bottom). Data are expressed as means ± SD of duplicate measurements and are represented by percentage changes from the original uninhibited antibody binding.

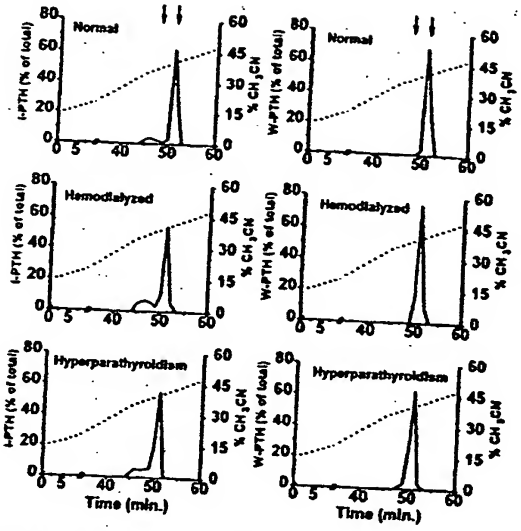


FIG. 4. HPLC profiles of immunoreactive PTH present in serum of a normal individual, a 1°-HPT patient, and a hemodialysis patient. Profiles were analyzed using the Nichols intact PTH IRMA and the whole PTH IRMA. Results are expressed as a percentage of the total immunoreactivity. A peak distinct from PTH(1-84) is detected by the intact PTH assay but not by the whole PTH(1-84) assay.

Assay validations using chromatographic fractionated samples

Figure 4 shows the two different immunoreactive PTH profiles with HPLC fractionated samples from 1 normal person, 1 patient with 1°-HPT, and one patient with 2°-HPT caused by chronic renal failure. The elution position of PTH(1-84) and of PTH(7-84), a prototype of those circulating non-(1-84) PTH fragments, also is indicated. Two immunoreactive peaks were detected in samples from all three groups using the Nichols intact PTH IRMA; the first peak corresponded to the aminoterminal truncated PTH with similar hydrophobicity and elution position as PTH(7-84) and the second one to the immunoreactive PTH(1-84), whereas, only one major immunoreactive peak corresponding to the elution position of PTH(1-84) was detected in all three samples using the newly developed whole PTH IRMA. Results of all HPLC runs are summarized in Table 1. There was a good agreement between the results of whole/intact PTH ratio and the amount of PTH(1-84) obtained by planimetric evaluation of the intact PTH HPLC profiles in the populations studied.

Sample stability for the whole PTH(1-84) measurement

The stability of whole PTH(1-84) was studied as follows: (1) in serum, EDTA plasma, and heparinized plasma; and (2) at 2–8°C and at room temperature (RT). The results indicated that: (a) whole PTH(1-84) in EDTA plasma and heparinized plasma is stable (<5% degradation) at 2–8°C or RT for at least 24 h; and (b) whole PTH(1-84) in serum, however, is only stable for 6 h at RT (>10% degradation) and for about 24 h at 2–8°C (Fig. 5). Additionally, a study of four times sample freeze/thaw showed that both serum and EDTA plasma were relatively stable with a <5% decrease in immunoreactivity.

Assay correlation and clinical evaluation

The normal range of whole PTH(1-84) was found to be 7–36 pg/ml (mean ± SD: 22.7 ± 7.2 pg/ml; n = 135) for EDTA plasma.

To study the correlation and difference between whole PTH(1-84) and conventional intact PTH levels in normal persons, 56 normal human EDTA plasma samples were measured at the same time with two different PTH assays, the newly developed whole PTH IRMA and the Nichols intact PTH IRMA. All the samples had measurable whole PTH(1-84) values. There were also measurable PTH values in all normal samples using the Nichols intact PTH IRMA. However, all intact PTH values measured by the Nichols PTH IRMA were higher than the whole PTH(1-84) values (Table 2) revealing an average of about 33% PTH fragments being comeasured with PTH(1-84) by intact PTH assay. Paired Student's t-test showed a significant difference ($p < 0.0001$) between the two sets of PTH values with these two PTH IRMAs (Fig. 6, bottom). The correlation of these two

TABLE 1. COMPARISON OF HPLC PROFILE RESULTS WITH WHOLE/INTACT PTH RATIOS IN NORMAL INDIVIDUAL, RENAL FAILURE, AND 1°-HPT PATIENTS

Groups	Ca^{2+} (mmol/liter)	Creatinine (μmol/liter)	Whole PTH (pg/ml)	Intact PTH (pg/ml)	Whole/intact PTH	HPLC results: Intact PTH (%) PTH(1-84)	HPLC results: Intact PTH (%) PTH fragment	Recovery (%)
Normals (n = 5)	2.25 ± 0.07	85.2 ± 6.6	24.1 ± 7.5	30.2 ± 9.5	0.8 ± 0.00	85.3 ± 1.5	14.7 ± 1.5	109 ± 9.8
Renal Failure (n = 5)	2.47 ± 0.12	864 ± 82	226 ± 185	337 ± 280	0.7 ± 0.07	65.3 ± 1.9	34.7 ± 1.9	70.3 ± 14.3
1°-HPT (n = 3)	2.58 ± 1.1	84 ± 23	53.5 ± 48.1	68.7 ± 42.0	0.69 ± 0.24	70.1 ± 9.7	29.9 ± 9.7	107.9 ± 4.5

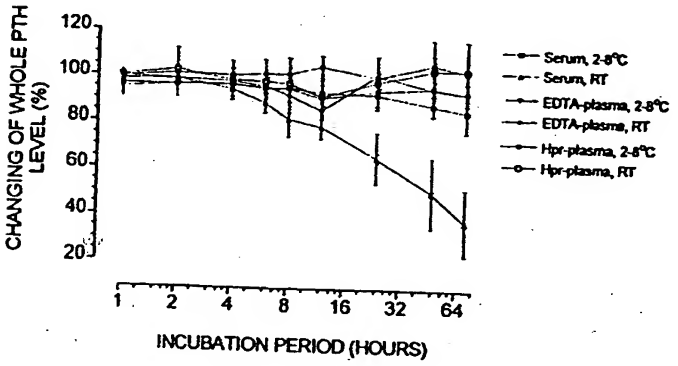


FIG. 5. Sample stability for the whole PTH(1-84) measurement. Data are expressed as means ± SD of duplicate measurements and are represented by percentage changes from the original concentrations.

groups of PTH values also was calculated (r = 0.923; slope = 1.456).

To ensure that this difference of PTH values was only caused by the specific antibody-antigen binding and not caused by differences in assay matrix or calibrators, different amounts of synthetic PTH(1-84) were spiked into several normal human sera with nondetectable PTH levels and measured with the previously mentioned two PTH assays. The result showed these two assays detect PTH(1-84) equally (r = 0.999; slope = 1.04; Fig. 6, top).

Human PTH values from a sample group of 318 uremic patients with ongoing hemodialysis also were determined with these two assays. The results showed that the PTH values displayed a heterogeneous distribution pattern in normal, below-normal, and elevated levels using both assays. The mean and median for the whole PTH(1-84) in this group also differed significantly from that obtained with the Nichols intact PTH assay ($p < 0.0001$; paired Student's t-test; Table 2). Figure 7 shows the correlation comparison of these two assays in the uremic patient group (r = 0.977; slope = 1.482). Samples from 165 patients with surgically confirmed 1°-HPT with parathyroid adenomas also were measured using the whole PTH IRMA (mean ± SD: 116.7 ± 129.6 pg/ml) and the Nichols intact PTH IRMA (mean ± SD: 200.3 ± 208.9 pg/ml). An effective differentiation of this patient group from normal persons was observed (Fig. 8). The overall clinical diagnostic sensitivity with a single sample PTH measurement was 93.9% (155/165) using whole PTH IRMA and 91.5% (151/165) using Nichols intact PTH IRMA.

The ratios of whole PTH to intact PTH or percentage of biologically active PTH(1-84) (pB%) to the total immunoreactive intact PTH were calculated for all 318 uremic patients and 165 1°-HPT patients. The results display an almost Gaussian distribution pattern from 20% to >90% in both patient groups (Fig. 9). This inconsistent pB% may be the result of variations in peripheral clearance of PTH or the glandular secretion of PTH(1-84) and its fragments.[20] This finding further indicates that currently available intact PTH values could not assess accurately the parathyroid function of patients.

DISCUSSION

The present report describes for the first time an immunoassay that measures only the biologically active whole PTH(1-84) without any cross-reactivity to PTH fragments, although current intact PTH immunoassays have been used and presumed to be specific for intact PTH for over 10 years. One study evaluated serum intact PTH levels in conjunction with histological analyses of iliac crest bone biopsy specimens.[7] It was found that serum intact PTH assays overestimate the presence and severity of PTH-

TABLE 2. COMPARISON OF INTACT PTH VALUES, WHOLE PTH(1-84) VALUES, AND pB% {(WHOLE PTH VALUE/INTACT PTH VALUE) × 100%} IN PATIENTS WITH UREMIA AND SURGICALLY PROVEN PRIMARY HPT WITH NORMAL PERSONS

	Uremic patients (n = 318)			*1°-HPT patients (n = 165)*			*Healthy controls (n = 56)*		
	Intact hPTH (pg/ml) [Nichols]	*Whole hPTH (pg/ml) [Scantibodies]*	*pB%* (%)*	*Intact hPTH (pg/ml) [Nichols]*	*Whole hPTH (pg/ml) [Scantibodies]*	*pB%† (%)*	*Intact hPTH (pg/ml) [Nichols]*	*Whole hPTH (pg/ml) [Scantibodies]*	*pB% (%)*
Minimum	3.4	1.1	15	32	23	22	13	8	35
Maximum	5230	1388	99	2000	909	98	67	37	88
Mean	442	254	55	200	117	59	41	26	65
Median	300	154	54	143	75	58	35	22	67
SD	515	223	15	209	130	16	25	16	16

* $p < 0.001$ for the ratio of whole/intact hPTH between uremic patients and healthy controls (two-tailed Mann-Whitney test).
† $p < 0.01$ for the ratio of whole/intact hPTH between 1°-HPT patients and healthy controls (two-tailed Mann-Whitney test).

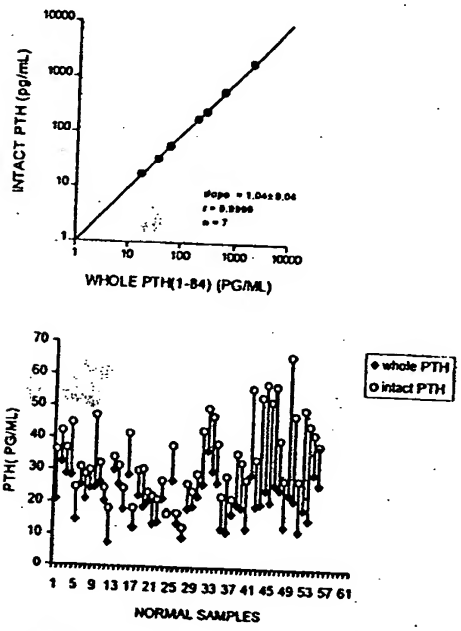


FIG. 6. Assay correlation studies of 56 normal persons (bottom; open circle, Nichols intact PTH IRMA; solid diamond, whole PTH IRMA) and from 7 artificial samples containing only whole PTH(1-84) (top). Data are expressed as means of duplicate measurements.

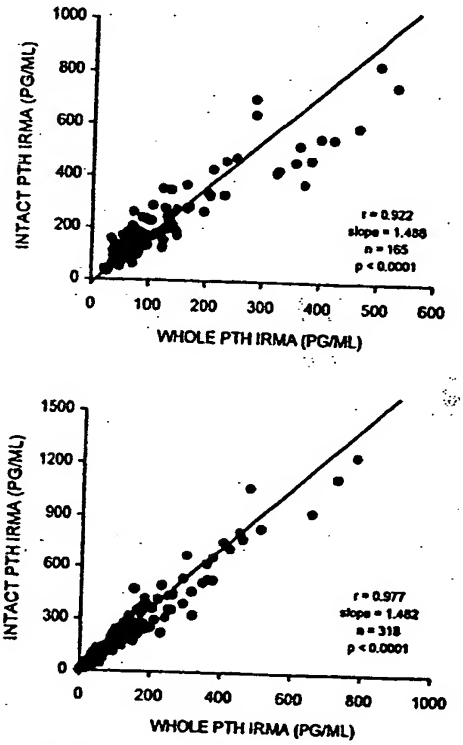


FIG. 7. Assay correlation study of 165 1°-HPT samples (top) and 318 uremic samples (bottom) using the Nichols intact PTH IRMA and the whole PTH IRMA. Paired Student's *t*-test was used for *p* value calculation.

mediated osseous abnormalities associated with uremia. Although at that time the reason for this overestimation was not elucidated, it might have been explained partially by this work in combination with recent studies.[10,11,21] It has been shown that the commercially available intact PTH assays measure both PTH(1-84) and non-PTH(1-84) fragments that are present in significant concentrations in the blood of uremic patients.[10] Therefore, these intact PTH assays are not truly intact PTH specific and the term "intact" is used inaccurately.

The specificity studies of the tracer antibody show that the newly developed anti-PTH(1-4) antibody is truly amino-terminal PTH specific. In fact, it is directed at the first amino acid of the aminoterminal polypeptide (Fig. 3), therefore, being able to bind to PTH(1-34) but not PTH(2-34), -(3-34), -(4-34), and -(5-34). By contrast, the tracer anti-

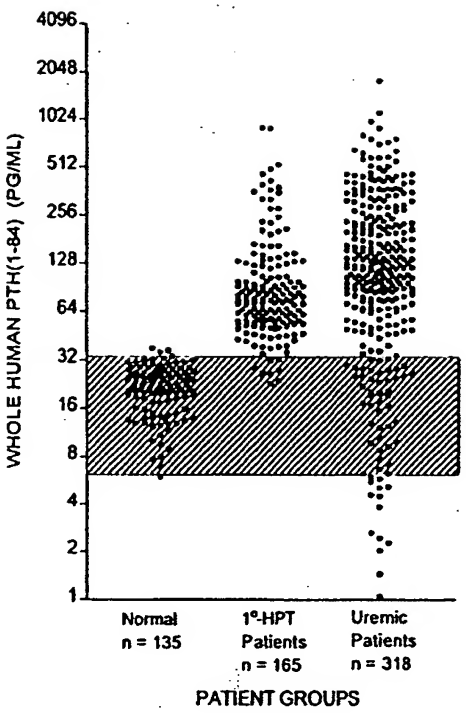


FIG. 8. Scatterplot of whole PTH(1-84) values in healthy controls and various patient groups. Shaded area indicates the plasma normal range (7–36 pg/ml) of whole PTH(1-84). The y axis is expressed by $\log_2$ scale. The whole PTH levels of 10 1°-HPT patients were located in the upper normal range and the overall diagnostic sensitivity was 93.9% (155/165).

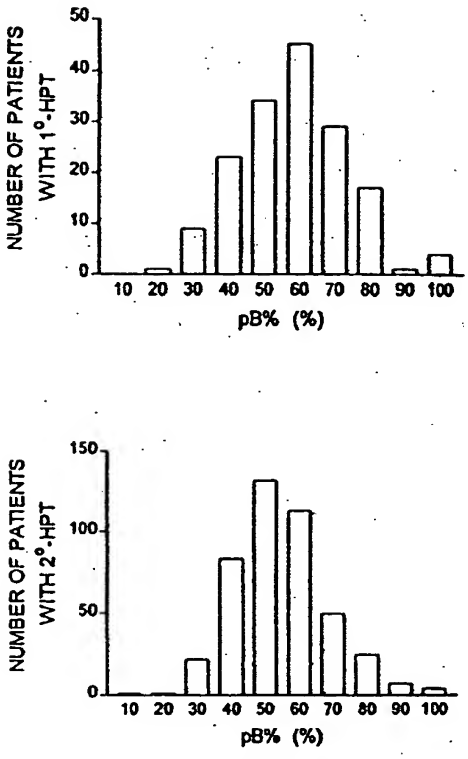


FIG. 9. Histogram showing the frequency distribution of the pB% in the pool of the total immunoreactive intact PTH value in patients with 1°-HPT (n = 165, top) and 2°-HPT (n = 318, bottom) of uremia.

body used in Nichols intact PTH IRMA is broadly PTH(1-34) specific and, therefore, cross-reacts with PTH(2-34), -(3-34), -(4-34), and -(5-34). It is the specificity of the tracer antibody used in this new whole PTH assay that ensures that this unique assay only detects the full-length PTH(1-84) without cross-reaction to any aminoterminally truncated PTH fragments. In theory, this assay also could detect carboxy-terminally slightly truncated PTH fragments, which should be the same for other intact PTH assays. Using the commercially available synthetic aminoterminally truncated PTH fragment, PTH(7-84) other intact PTH assays (Incstar, Diagnostic System Laboratory, Diagnostic Product Corp.) show variable cross-reactivity of 60–80% from assay to assay.[22] The whole PTH IRMA was thoroughly designed and developed in a coated bead format and single incubation step. It is easy to perform and presents a clinically adequate measurement range of 1–2300 pg/ml with acceptable assay performance characteristics, including linearity, sample spiking recovery, and intra-/interassay precision.

The study of the chromatographically fractionated serum samples from normal population and patients with either 1°-HPT or 2°-HPT further shows that there are two forms of PTH or immunoreactive peaks detected by the Nichols intact PTH IRMA. The first immunoreactive peak corresponds to non-PTH(1-84) fragments migrating on HPLC to a similar position as PTH(7-84) and the second peak corresponds to the full-length PTH(1-84).[10] However, when the same samples were measured with the whole PTH IRMA, only one immunoreactive peak was detected corresponding to the full-length PTH(1-84). Comparing the specificity of the antibodies used in these two assays, it is quite obvious that the non-PTH(1-84) corresponds to aminoterminally truncated PTH fragments. Moreover, these HPLC fractionated patient sample measurements further show that these aminoterminally truncated polypeptides are present in significant amounts not only in uremic patients, but also in the normal population and in patients with 1°-HPT (Fig. 4; Table 1). The exact molecular structure of these PTH fragments should be further determined by isolating and analyzing their amino acid sequences using pools of patient serum samples.

The correlation study of whole PTH IRMA to Nichols intact PTH IRMA from samples that contain only synthetic PTH(1-84) indicates that the two assays are nearly equivalent in their detection of PTH(1-84) (Fig. 6, top). However, when clinical samples from a normal population group and patients with 1°-HPT or 2°-HPT were used for the study, significant differences with higher intact than whole in the

absolute PTH values were found ($p < 0.0001$, paired t-test) in all three groups (Figs. 6 and 7; Table 2).

The clinical significance of this newly developed whole PTH IRMA was shown in three population groups. The normal range of whole PTH(1-84) was 7–36 pg/ml for samples of EDTA plasma. Samples of EDTA plasma are preferred for whole PTH measurement because the hormone appears to be more stable in EDTA plasma than in the serum (Fig. 5). There is an unexpected distinction in whole PTH(1-84) levels of patients with 1°-HPT from the normal population with an overall diagnostic sensitivity of 93.9% ($n = 165$) in this study. A diagnostic sensitivity of 91% also was found with Nichols intact PTH IRMA in this study. However, Kao et al.[23] evaluated 361 patients with surgically proven 1°-HPT in whom intact PTH had been determined with an immunochemiluminometric assay and found 45 patients to have an intact PTH value below the upper limit of normal. Endres et al.[24] also reported that only 21 of 29 cases of 1°-HPT had values above the normal level when the Nichols Allégro intact PTH assay was used. These early studies indicated a diagnostic sensitivity of intact PTH assay of about 72.4–87.5% only. Most recently, Silverberg et al.[25] reported a prospective clinical validation using whole PTH assay, Nichols intact PTH assay, and a midregional PTH competitive assay. In her study, a well-defined group of patients with mild 1°-HPT was chosen and the clinical diagnostic sensitivities were 96% for whole PTH assay, 76% for intact PTH assay, and 54% for a midregional PTH assay. Significant statistical differences were found between each assay in this study. Whole PTH(1-84) values from 318 uremic patients displayed a heterogeneous distribution pattern with both normal and elevated levels.

This study has shown that there is no consistent percentage of aminoterminally truncated PTH fragments (Fig. 9; Table 2). It is inconsistent percentage of aminoterminally truncated PTH fragments among patients with HPT that could easily give rise to two previously unforeseen major problems in the clinical decisions based on available intact PTH assays for evaluating the function of the parathyroid glands. First, because most intact PTH assays have >60% cross-reaction[10] to the PTH fragments and the ratio of whole PTH/intact PTH or the pB% is not consistent even in patients in the same disease condition, the parathyroid function will always be overestimated and inconsistently estimated in different degrees by intact PTH assays measuring both the full-length whole PTH(1-84) and its aminoterminally truncated fragments. Second, because of the significantly different molar rates of cross-reactivity of commercially available intact PTH assays, interlaboratory discordance of PTH levels have been observed from the use of different intact PTH assays. Theoretically, the aminoterminally truncated PTH fragment is a naturally produced polypeptide, which is able to bind to PTH/PTH-related protein (PTHrP) receptors. One preliminary in vivo study with parathyroidectomized rats showed an 80% decreased calcemic response for a 1:1 molar ratio of infused PTH(7-84) and PTH(1-84) compared with PTH(1-84) alone.[26] The biological importance of these aminoterminally truncated fragments that have been shown to act as PTH antagonist or inhibitor appears to regulate eventually the sensitivity of PTH/PTHrP receptors and warrants further investigation. These PTH fragments also could be ligands for a thus far unisolated receptor for the carboxy-terminal part of PTH. However, whether this receptor plays a role in the regulation of calcium metabolism is not known.[27]

In summary, a novel IRMA was developed that only detects biologically active whole PTH(1-84) without cross-reaction to the aminoterminally truncated PTH fragments. The assay uses only a single incubation procedure. The PTH(1-84) specificity of the new assay was defined by tracer antibody evaluation, cross-reactivity experiments, and measurements of HPLC fractionated patient samples. With this whole PTH IRMA, we first showed that previously described non-(1-84) PTH fragments[10] should be aminoterminally truncated. The presence of these aminoterminally truncated PTH fragments was shown not only in uremic patients, but also in 1°-HPT patients and normal persons. Moreover, the percentage concentration of the biologically active whole PTH(1-84) in the pool of total immunoreactive intact PTH is significantly variable from patient to patient, even in patients with the same type of HPT and, thus, it is impossible to interpret biologically active PTH levels with current intact PTH assays. The new whole PTH IRMA is clinically significant in differentiating patients with 1°-HPT and 2°-HPT from the normal population in measuring PTH(1-84) exclusively. Because of the immunological heterogeneity of circulating PTH, this new assay model could be applied as a more meaningful and standardized method for the measurement of biologically active and hence clinically significant PTH.

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